CARBON TETRACHLORIDE INCREASES INTRACELLULAR CALCIUM IN RAT LIVER AND HEPATOCYTE CULTURES

1986

LONG

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
JUL 1986	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Carbon Tetrachloride Increases Intracellular Calcium in Rat Liver and Hepatocyte Cultures	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Uniformed Services University Of The Health Sciences Bethesda, MD 20814**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**162**	

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
F. EDWARD HÉBERT SCHOOL OF MEDICINE
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

GRADUATE AND
CONTINUING EDUCATION

TEACHING HOSPITALS
WALTER REED ARMY MEDICAL CENTER
NAVAL HOSPITAL, BETHESDA
MALCOLM GROW AIR FORCE MEDICAL CENTER
WILFORD HALL AIR FORCE MEDICAL CENTER

APPROVAL SHEET

Title of Thesis: Carbon Tetrachloride Increases Intracellular Calcium in Rat Liver and Hepatocyte Cultures

Name of Candidate: Rochelle Miller Long
Doctor of Philosophy Degree
May 12, 1986

Thesis and Abstract Approved:

Committee Chairperson	May 12, 1986 Date
Committee Member	12 May 1986 Date
Committee Member	12 May 1986 Date
Committee Member	12 May 1986 Date
Committee Member	May 12, 1986 Date

The author hereby certifies that the use of any copyrighted material in the dissertation manuscript entitled:

"Carbon Tetrachloride Increases Intracellular Calcium in Rat Liver and Hepatocyte Cultures"

beyond brief excerpts is with the permission of the copyright owner, and will save and hold harmless the Uniformed Services University of the Health Sciences from any damage which may arise from such copyright violations.

Rochelle Miller Long

Rochelle Miller Long
Department of Pharmacology
Uniformed Services University
of the Health Sciences

ABSTRACT

Title of Dissertation: Carbon Tetrachloride Increases Intracellular Calcium in Rat Liver and Hepatocyte Cultures

Rochelle Miller Long, Doctor of Philosophy, 1986

Dissertation directed by: Leon Moore, Ph.D., Associate Professor, Department of Pharmacology

Sequestration of ionized calcium (Ca^{++}) by rat liver endoplasmic reticulum (ER) is inhibited following in vivo administration of the hepatotoxin carbon tetrachloride (CCl_4). Consequently, hepatic cytosolic Ca^{++} may rise to supraphysiologic levels, perhaps leading to excessive stimulation of Ca^{++}-sensitive processes occurring within liver cells. The hypothesis examined was whether cytoplasmic Ca^{++} concentrations are elevated in rat liver and hepatocyte cultures exposed to CCl_4. Ca^{++} levels were determined indirectly via the Ca^{++}-dependent conversion of glycogen phosphorylase to the a form, and directly with the fluorescent Ca^{++}-indicator compound quin2. Other biochemical alterations produced by CCl_4 were also monitored in order to relate Ca^{++} increases to the development of hepatotoxicity. Within half an hour after administration of CCl_4 to rats (1.5 ml/kg body weight), liver phosphorylase a levels increased (136% of control), liver glycogen concentrations decreased (66% of control), and ER Ca^{++} pump activity was inhibited (35% of control). These effects persisted through 24 hours. Hepatic glucose-6-phosphatase activity did not decrease until two hours (71% of control), and 5′-nucleotidase activity never changed. By eight hours, serum glutamic-pyruvic transaminase activity and total liver calcium levels were elevated (939% and 450% of control, respectively).

In cultured hepatocytes exposed to CCl_4 (3 mM) for five minutes,

phosphorylase *a* activity increased (392% of control) and ER Ca^{++} pump activity decreased (19% of control). These effects were dose-dependent between 0.3 and 3 mM CCl_4. From a calibration curve relating cellular phosphorylase *a* activity to Ca^{++} concentrations, it was estimated that cytosolic Ca^{++} rose to micromolar levels for at least 60 minutes in hepatocytes exposed to CCl_4. Experiments with quin2 confirmed Ca^{++} increases of this magnitude. In contrast, the alpha-adrenergic agent phenylephrine (a non-hepatotoxin) stimulated phosphorylase *a* activity to a lesser extent, and this activation was not sustained for greater than 10 minutes. In hepatocytes exposed to CCl_4, extracellular Ca^{++} was not required for elevated phosphorylase *a* activity, suggesting that Ca^{++} was released from internal stores. Increased phosphorylase *a* activity was not accompanied by generation of cyclic AMP (an alternative route for phosphorylase activation). Hepatocyte glucose-6-phosphatase activity was not inhibited until 20 minutes (71% of control), and 5′-nucleotidase activity was not affected. These results demonstrate that CCl_4 exposure produces sustained elevation of cytosolic Ca^{++} concentrations in liver cells *in vivo* and *in vitro*. Elevated hepatic Ca^{++} occurs with a time course that would allow Ca^{++} to play a role in CCl_4-induced hepatotoxicity.

CARBON TETRACHLORIDE INCREASES INTRACELLULAR CALCIUM IN RAT LIVER AND HEPATOCYTE CULTURES

by

Rochelle Miller Long

Dissertation submitted to the Faculty of the Department of Pharmacology
Graduate Program of the Uniformed Services University of the
Health Sciences in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy 1986

DEDICATION

To J. B. L.

ACKNOWLEDGEMENTS

Thanks to Beth L. DiGiulian, Kristen C. Zeller, and Cynthia L. Wallace for providing many excellent hepatocyte preparations for these experiments. I valued both their technical assistance and their great company in the lab.

Many thanks to Leon "Dr. Mac" Moore. It was only with his guidance that I was able to execute the experiments described in this dissertation. He also made graduate school a lot of fun along the way. I'd almost do it again.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AMP	adenosine 5′-monophosphate
ATP	adenosine 5′-triphosphate
cAMP	adenosine 3′:5′-cyclic monophosphate
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-(beta-aminoethyl ether)-N,N′-tetraacetic acid
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
NADH	beta-nicotinamide adenine dinucleotide, reduced
NADPH	beta-nicotinamide adenine dinucleotide phosphate, reduced
Quin2	2-bis(carboxymethyl)-amino-5-methylphenoxy-methyl-6-methoxy-8-bis(carboxymethyl)-aminoquinoline
SKF-525A	2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride
Tris	tris(hydroxymethyl)-aminomethane

INTRODUCTION

CARBON TETRACHLORIDE: A PROTOTYPE HEPATOTOXIN

Carbon tetrachloride (CCl_4) is an historically important hepatotoxin that has been investigated since before the turn of the century. CCl_4 was first used as an anesthetic (introduced in 1847), and later as an antihelminthic (beginning 1921). By the 1940's, the hepatic fat accumulation caused by CCl_4 was well described in the literature. Early efforts to determine the mechanism of CCl_4 toxicity focused on lipid metabolism. As knowledge of the physiology and biochemistry of the cell progressed, CCl_4 was subsequently found to perturb many sites within the liver cell, including the endoplasmic reticulum (ER), mitochondria, and lysozomes. A spectrum of toxic effects occurs at the cellular level following administration of CCl_4 to rats (reviewed by Recknagel, 1967). Within half an hour, the endoplasmic reticulum changes in appearance as the cisternae become dilated, polyribosomes disaggregate, protein synthesis is inhibited, and some microsomal enzyme activities (ER Ca^{++} pump and mixed function oxidase activities) are depressed. Concomitantly, lipid metabolism is disturbed and fat accumulates in the liver cell, in part as a result of the depression in synthesis of lipid acceptor proteins. By two to four hours, permeability of the mitochondria is affected, and these organelles swell as electrolytes accumulate and oxidative phosphorylation becomes uncoupled. Stores of liver glycogen are significantly depleted by this time, and another microsomal enzyme, glucose-6-phosphatase, is now inhibited. By five to ten hours, lysozomes have become structurally deranged and rupture. After eight to ten hours, intracellular enzymes can be found in the plasma, total cell calcium (ionized and complexed) levels are elevated many-fold, and there

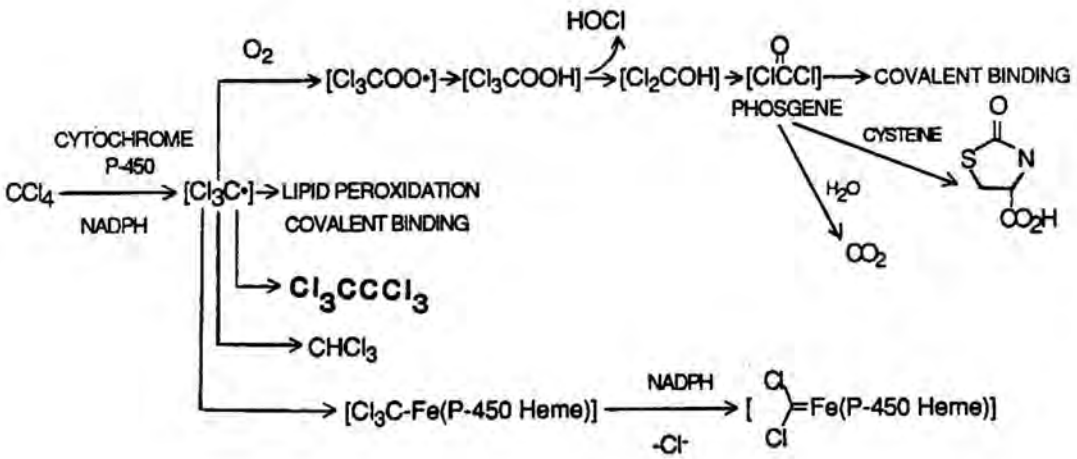
is histological evidence of coagulative necrosis. It is not yet known at which stage in this continuum of toxic effects the irreversible point is reached and permanent liver damage becomes unavoidable. Many studies have been directed towards identifying the earliest crucial event on the pathway to hepatotoxicity. Selected experiments are described in more detail below.

Metabolism of Carbon Tetrachloride

CCl_4 is reductively metabolized by the cytochrome P-450-dependent mixed function oxidase system to the very reactive trichloromethyl free radical. Chloroform, carbon dioxide, and hexachloroethane are subsequently generated from this reactive molecule (Figure 1). Another reactive metabolite, phosgene, is detected by trapping as an adduct of cysteine. These metabolic pathways have been demonstrated to exist both *in vivo* and *in vitro* under conditions of varying oxygen tensions, and they may not all occur simultaneously (reviewed by MacDonald, 1983). Use of metabolic inducers and inhibitors can affect the severity of hepatic damage caused by CCl_4. Pretreatment of rats with phenobarbital, the classic inducer of the mixed function oxidase system, enhances the toxicity of CCl_4 (Garner and McLean, 1969). Pretreatment with Aroclor 1254, a mixture of polychlorinated biphenyls that causes broad spectrum induction, enhances CCl_4 toxicity in rats as well (Carlson, 1975). Isopropanol pretreatment, which selectively induces specific forms of cytochrome P-450 (Ueng *et al.*, 1983), markedly exacerbates the toxicity of CCl_4 (Traiger and Plaa, 1971). Aliphatic ketones such as 2,5-hexanedione are also potent potentiators of chlorinated hydrocarbon toxicity (Jernigan and Harbison, 1982). In contrast, pretreatment of rats with 3-methylcholanthrene, an inducer of cytochrome P-448 in rat

Figure 1. Metabolic Activation of Carbon Tetrachloride

Generation of trichloromethyl radical, phosgene, hexachloroethane, chloroform and other reactive species by cytochrome P-450-dependent metabolism of carbon tetrachloride (adapted from MacDonald, 1983).


HOCl
O2
[Cl3COO•]→[Cl3COOH]→[Cl2COH]→[ClCCl]→COVALENT BINDING
O
PHOSGENE
CYTOCHROME
P-450
CCl4
NADPH
[Cl3C•]→LIPID PEROXIDATION
COVALENT BINDING
CYSTEINE
O
S
N
CO2H
H2O
CO2
Cl3CCCl3
CHCl3
[Cl3C-Fe(P-450 Heme)]
NADPH
-Cl-
Cl
Cl
=Fe(P-450 Heme)]

liver, affords protection from CCl_4-induced hepatic injury (Suarez et al., 1972). It has been known for some time that CCl_4 causes a decrease in total cytochrome P-450 content of liver (Sasame et al., 1968), and it has recently been demonstrated that a specific form of cytochrome P-450, induceable by phenobarbital but not by 3-methylcholanthrene, is susceptible to destruction by CCl_4 (Head et al., 1981). Thus, the activation of CCl_4 to a toxic moiety clearly depends upon metabolism by one or more specific forms of cytochrome P-450.

Proposed Mechanisms of Carbon Tetrachloride Toxicity

Several mechanisms for CCl_4-induced liver injury have been proposed over the past 25 years. The trichloromethyl radical itself and the derived trichloromethyl dioxide radical can induce lipid peroxidation (the self-propagating oxidative destruction of polyunsaturated lipids) at the endoplasmic reticulum, destroying enzymes located there (reviewed by Recknagel and Glende, 1973). These reactive species, and phosgene as well, can also covalently bind to microsomal lipids and proteins (reviewed by MacDonald, 1983). Calcium (ionized and complexed) accumulates in necrotic liver tissue from animals given CCl_4 (Reynolds, 1963 and 1964). Lipid peroxidation, covalent binding, and altered Ca^{++} fluxes have been extensively investigated in order to distinguish their respective contributions to CCl_4 toxicity. Other mechanisms explaining CCl_4-induced liver damage have been briefly considered but eventually dismissed, including altered blood flow, mobilization of catecholamines, and depression of protein synthesis (reviewed by Recknagel, 1967).

Lipid Peroxidation

Lipid peroxidation is wildly destructive of cell membranes and can certainly cause extensive cellular injury. Recknagel and coworkers extensively characterized lipid peroxidation that occurs *in vivo* and *in vitro* (microsomes) following CCl_4 exposure (reviewed by Recknagel and Glende, 1973). Conjugated dienes (a measure of lipid peroxidation) were detected selectively in hepatic microsomal lipids within 15 minutes after rats were given a moderate dose of CCl_4. In microsomes, when NADPH was eliminated to prevent metabolism by cytochrome P-450, evidence of lipid peroxidation was diminished and membranes were protected from CCl_4's effects. Scavengers of free radicals and antioxidants such as alpha-tocopherol (vitamin E), diphenyl-p-phenylenediamine, and selenium partially protected liver from CCl_4-induced injury (reviewed by Plaa and Witschi, 1976). However, Klaassen and Plaa (1969) found that low doses of CCl_4 *in vivo* that produced biochemical and morphological changes in rat liver did not cause lipid peroxidation. Additionally, other halogenated hydrocarbons, including 1,1- dichloroethylene (vinylidene dichloride), chloroform, ethylene dibromide, and halothane, and other toxic agents such as thioacetamide and dimethylnitrosamine, caused little or no lipid peroxidation *in vivo* and under physiological conditions *in vitro*, yet these agents produced lesions at the cellular level similar to those observed after CCl_4 (reviewed by Plaa and Witschi, 1976). These arguments suggested that lipid peroxidation might not be a prerequisite for hepatotoxicity.

The role of lipid peroxidation in CCl_4 toxicity was investigated in suspensions of hepatocytes, and these studies yielded mixed results. Pretreatment of cells with promethazine (used as an antioxidant) completely prevented and SKF-525A (inhibitor of drug metabolizing enzymes)

partially prevented lipid peroxidation, but both agents did not affect inhibition of lipoprotein secretion when low concentrations of CCl_4 vapors were used (Gravela et al., 1979, Poli et al., 1979). Thus it was concluded that lipid peroxidation was not required for the inhibition of protein secretion that occurs after CCl_4. These workers found that lipid peroxidative damage was correlated with decreases in ER glucose-6-phosphatase activity, but not with the loss of cytochrome P-450 after CCl_4 (Poli et al., 1981). Others have found that promethazine and dibenamine (used as antioxidants) and SKF-525A could not protect from the toxic effects of relatively high concentrations of CCl_4 vapors, as measured by altered metabolic parameters (ureogenesis, lactate/pyruvate ratio), and release of potassium and intracellular enzymes (Stacey and Priestly, 1978a). These investigators noted that incubation of cells at 0-4° C did afford protection. Smith et al. (1982) found that when lipid peroxidative damage was measured by a newer, more sensitive technique (ethane production) in hepatocyte suspensions, CCl_4 was capable of causing significant lipid peroxidation in hepatocytes that correlated with losses in cell viability, as measured by NADH exclusion.

Covalent Binding

Castro and coworkers characterized the covalent binding of molecular fragments that occurs following in vivo CCl_4 administration. They observed tissue-specific (Castro et al., 1972) and species-related (Diaz-Gomez et al., 1975) metabolic activation and consequent covalent binding of ^{14}C from CCl_4 to cellular lipids that correlated with the development of necrosis. They described ^{14}C adducts covalently bound to heme following CCl_4 and consequent destruction of functional cytochrome P-450 (Fernandez et al., 1982). However, loss of cytochrome P-450 alone

does not constitute sufficient cellular damage for cell death to result. It was not known what cellular macromolecule was the crucial target that was inactivated by covalently bound fragments arising from CCl_4.

Covalent binding of CCl_4-derived products to cellular macromolecules was evaluated in cultures of hepatocytes. Time- and concentration-dependent covalent binding of ^{14}C from CCl_4 was described that correlated with the release of intracellular enzymes (Chenery et al., 1981). Cunningham et al. (1981) observed that covalent binding in suspensions of cells was greater under nitrogen- than oxygen-containing atmospheres, consistent with the observation that CCl_4 is reductively bioactivated by cytochrome P-450-dependent enzymes (Pohl et al., 1984). Paradoxically, when hepatocytes were prepared from rats that had been pretreated with phenobarbital (inducer of mixed function oxidase enzymes), no enhancement of covalent binding after CCl_4 was observed (Cunningham et al., 1981). Casini and Farber (1981) found evidence of both lipid peroxidation and covalent binding in hepatocyte cultures after CCl_4, and pretreatment with SKF-525A decreased both of these parameters.

Thus, diverse aspects of CCl_4 toxicity, including lipid peroxidation, covalent binding, inhibition of lipoprotein secretion, loss of endoplasmic reticulum enzymes glucose-6-phosphatase and cytochrome P-450, and release of intracellular enzymes were observed both in vivo and in vitro. However, despite numerous extensive investigations with different liver preparations (intact rats, hepatocytes, microsomes), causal relationships proposed between these aspects of CCl_4 toxicity varied greatly, perhaps depending upon the many possible experimental conditions. Conditions such as oxygen tension or the presence of ferric

iron in microsomal preparations can be manipulated in order to implicate or exclude either covalent binding or lipid peroxidation (Fander et al., 1982, Waller et al., 1983), and so the relative contributions of either of these mechanisms to CCl_4 toxicity remains a cause for debate.

Altered Calcium Fluxes

In 1966, Magee proposed that changes in ion transport and membrane permeability could be causally related to hepatotoxic injury (reviewed by Magee, 1966). Judah et al. (1964) suggested that shifts in membrane bound Ca^{++} within the liver cell could initiate irreversible actions leading to cellular necrosis. A wide variety of hepatotoxic agents, including CCl_4, thioacetamide, dimethylnitrosamine, galactosamine, and liver ischemia greatly increase total liver calcium content (reviewed by Farber, 1980). Reynolds reported that following administration of CCl_4 to rats, total liver cell calcium (ionized and complexed) increased slightly within two hours, returned to normal levels by four through eight hours, and rose dramatically between eight and 12 hours, until calcium reached 20 times normal levels by 48 hours (Reynolds, 1963 and 1964). Farber and associates proposed that an influx of extracellular Ca^{++} was a final common pathway in the development of liver necrosis (reviewed by Farber, 1980). Others claimed that an early disruption in intracellular Ca^{++} homeostasis could be a crucial event in the development of hepatotoxicity (reviewed by Recknagel, 1983), based upon observations of the rapid inhibition of microsomal Ca^{++} sequestration produced by CCl_4 and other hepatotoxic agents including 1,1-dichloroethylene, chloroform, and bromotrichloromethane (Moore, 1980, Lowrey et al., 1981).

Several investigators have studied the role of extracellular Ca^{++}

in CCl_4 toxicity in isolated hepatocytes. Casini and Farber (1981) reported that the extent of CCl_4-induced cell death in cultured hepatocytes, as measured by the proportion of cells that failed to exclude the dye trypan blue, depended on the Ca^{++} concentration of the medium. They observed that when hepatocytes were isolated from phenobarbital-pretreated rats, Ca^{++}-dependent cell killing by CCl_4 was potentiated. When SKF-525A was included in the medium, cell death was prevented. However, cell killing varied greatly (8% to 78%) between CCl_4-treated controls for these experiments, casting some doubt on results obtained with metabolic pretreatments. Chenery et al. (1981) examined intracellular enzyme release in hepatocyte cultures exposed to CCl_4 in Ca^{++}-containing and Ca^{++}-free media. They found that Ca^{++} in the medium was not required for CCl_4 toxicity. Smith et al. (1981) observed that CCl_4 was more toxic to freshly isolated hepatocytes in the absence rather than the presence of extracellular Ca^{++}, when toxicity was evaluated by trypan blue or NADH exclusion. Mixed results were obtained for a variety of other hepatotoxins. Some investigators described a requirement for medium Ca^{++} for cell killing and protection from cell death in the absence of Ca^{++} (Schanne et al., 1979, membrane-active toxins; Acosta and Sorenson, 1983, metals), while others found no protection from chemically-induced injury by omission of medium Ca^{++} (Smith et al., 1981, bromobenzene, ethylmethanesulfonate; Stacey and Klaassen, 1982, metals, amphotericin B). Fariss and Reed (1985) demonstrated that chemically-induced injury to hepatocytes could be prevented or potentiated by extracellular Ca^{++}, depending on the experimental conditions. Thus, a role for extracellular Ca^{++} was not definitively established. It was not known whether an influx of extracellular Ca^{++} was the cause

of, or resulted from, hepatotoxic damage.

Pencil et al. (1982) first considered the effects of CCl_4 on internal Ca^{++} homeostasis in suspensions of hepatocytes. When cells were exposed to very low concentrations of CCl_4 vapors, ER Ca^{++} pump activity was halved within ten minutes after CCl_4 addition. Inhibition of ER Ca^{++} pump activity correlated with appearance of covalently bound products derived from CCl_4. These investigators later found that inhibition of the ER Ca^{++} pump was not required for inhibition of lipoprotein secretion caused by CCl_4 (Pencil et al., 1984). Others found that inhibition of ER Ca^{++} sequestration was selective, and mitochondrial Ca^{++} accumulation was unaffected by low doses of CCl_4 at early times in hepatocytes (Santone and Acosta, 1985). To date, no one else has investigated possible CCl_4-induced alterations in intracellular Ca^{++} homeostasis in liver cells. No studies have considered whether cytoplasmic Ca^{++} concentrations might be elevated as a consequence of the ER Ca^{++} pump inhibition caused by CCl_4.

CALCIUM HOMEOSTASIS IN RAT LIVER CELLS

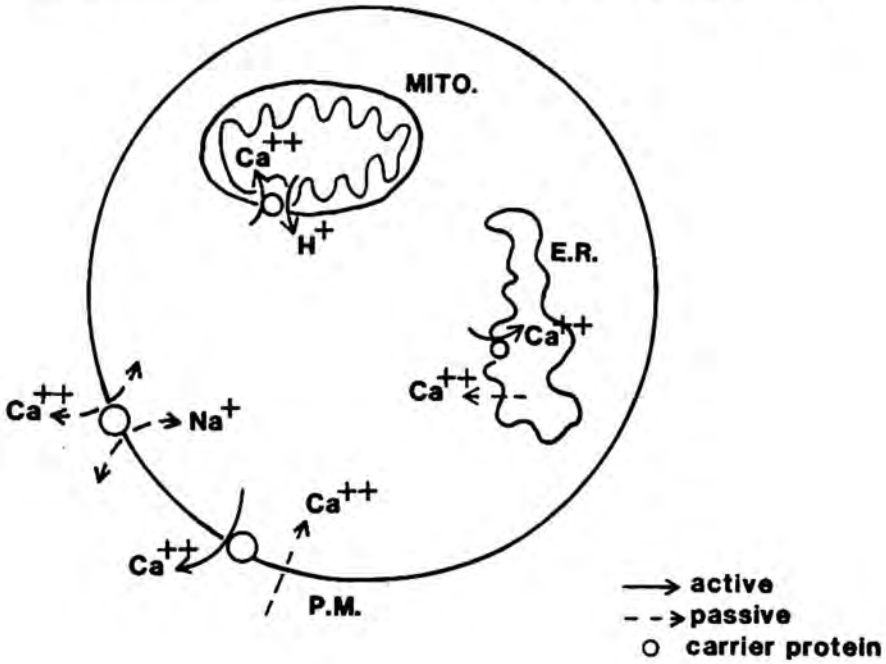
Regulation of Cell Calcium Concentrations

Intracellular ionized calcium is maintained at much lower concentrations than extracellular ionized calcium, which is on the order of one mM. In resting liver cells, intracellular Ca^{++} concentrations are reported to range from 100 to 200 nM (Murphy et al., 1980, Charest et al., 1983). There are several mechanisms responsible for controlling cytoplasmic Ca^{++} concentrations, and any of these sites could be susceptible to the toxic actions of CCl_4. Ca^{++} extruding or sequestering mechanisms are located at the endoplasmic reticulum, plasma membrane, and mitochondria (Figure 2). All work by active processes to

Figure 2. Regulation of Calcium Homeostasis in the Hepatocyte

Energy-dependent calcium sequestering mechanisms are located at the endoplasmic reticulum (ER), the plasma membrane (PM), and the mitochondria (Mito) of the hepatocyte.

CALCIUM HOMEOSTASIS IN THE HEPATOCYTE



restore and maintain the gradient of Ca^{++} concentrations across cellular membranes (reviewed by Akerman, 1982). The Ca^{++} pump at the endoplasmic reticulum hydrolyzes $Mg^{++}ATP$, transports Ca^{++} into the reticular lumen, and is opposed by a passive leak of Ca^{++} out of the organelle (Moore et al., 1975). This pump is analagous to, but probably less specialized than, that found at sarcoplasmic reticulum of muscle cells. The plasma membrane has an ATP-dependent Ca^{++} pump that is opposed by a slow influx of Ca^{++} (VanRossum, 1970). The plasma membrane also has a reversible Ca^{++}/Na^{+} exchanger linked to the Na^{+} electrochemical gradient formed by $Na^{+}/K^{+}ATPase$ (Bernstein and Santacana, 1985). Mitochondria have a Ca^{++} sequestering mechanism that can utilize ATP or be driven by the proton gradient formed during mitochondrial respiration (Lehninger et al., 1967). Although mitochondria provide a relatively high capacity Ca^{++} sink, the affinity of this pump for Ca^{++} is much less that of the Ca^{++} pumps found at endoplasmic reticulum and plasma membranes (K_m on the order of 10^{-5} M vs. 10^{-6} M for the latter two pumps), thus mitochondria do not normally accumulate Ca^{++} under physiologic cell conditions (Carafoli, 1982). The surface area of endoplasmic reticulum is 40 times as great as the surface area of plasma membrane in the hepatocyte (Weibel et al., 1969), therefore the ER Ca^{++} pump may be the major determinant quantitatively of cell Ca^{++} concentrations. When any of these Ca^{++} transporting systems is disrupted, it becomes possible that normal Ca^{++} homeostasis will be lost and the cell will suffer as a result.

Potential Consequences of Elevated Cytosolic Calcium

Normally, Ca^{++} fluxes in liver cells are tightly regulated. When hepatocyte suspensions are exposed to Ca^{++}-mobilizing hormones such as

epinephrine, vasopressin, or angiotensin II, the intracellular transducer myo-inositol 1,4,5-trisphosphate causes release of Ca^{++} from endoplasmic reticulum (Joseph et al., 1984, Burgess et al., 1984). Sufficient Ca^{++} is released to raise cytoplasmic Ca^{++} levels to 900 nM for at least three to five minutes. An influx of extracellular Ca^{++} may maintain cytosolic Ca^{++} at intermediate levels (400 to 600 nM) for several minutes more, after which time Ca^{++} returns to basal levels (around 200 nM) (Joseph et al., 1985, Binet et al., 1985). These transient Ca^{++} increases briefly activate Ca^{++}-dependent metabolic enzymes such as protein kinases, usually via calmodulin (reviewed by Williamson et al., 1981).

In order for Ca^{++} to act as an intracellular toxin, Ca^{++} concentrations must become elevated in an uncontrolled fashion. Ca^{++} concentrations could become supraphysiologic either in magnitude or in duration. In some manner, normally tightly regulated Ca^{++} fluxes must be disturbed for hepatotoxicity to develop. Many Ca^{++}-responsive enzymes could be overstimulated by supraphysiologic Ca^{++} concentrations, including protein kinases, protein phosphatases, phospholipases, proteases, nucleases, and others (reviewed by Shier, 1982). Phospholipases A_2 and C are leading candidates to cause cellular injury if inappropriately activated, because they regulate membrane composition and turnover (Scott, 1984). Accelerated degradation of cellular phospholipids accompanying elevated intracellular Ca^{++} has been demonstrated in ischemic liver injury (Chien et al., 1978). Lysolecithin and arachidonate released by actions of phospholipase A_2 can act as a natural detergents to activate enzymes and disrupt cell membranes. Proteases can destroy essential biosynthetic enzymes, transport proteins, or

cellular structural elements. Nucleases can hydrolyze information-containing nucleic acids. Uncontrolled activation of any or all of these cellular enzymes potentially can constitute an effective cytotoxic mechanism.

DETERMINATION OF INTRACELLULAR CALCIUM CONCENTRATIONS

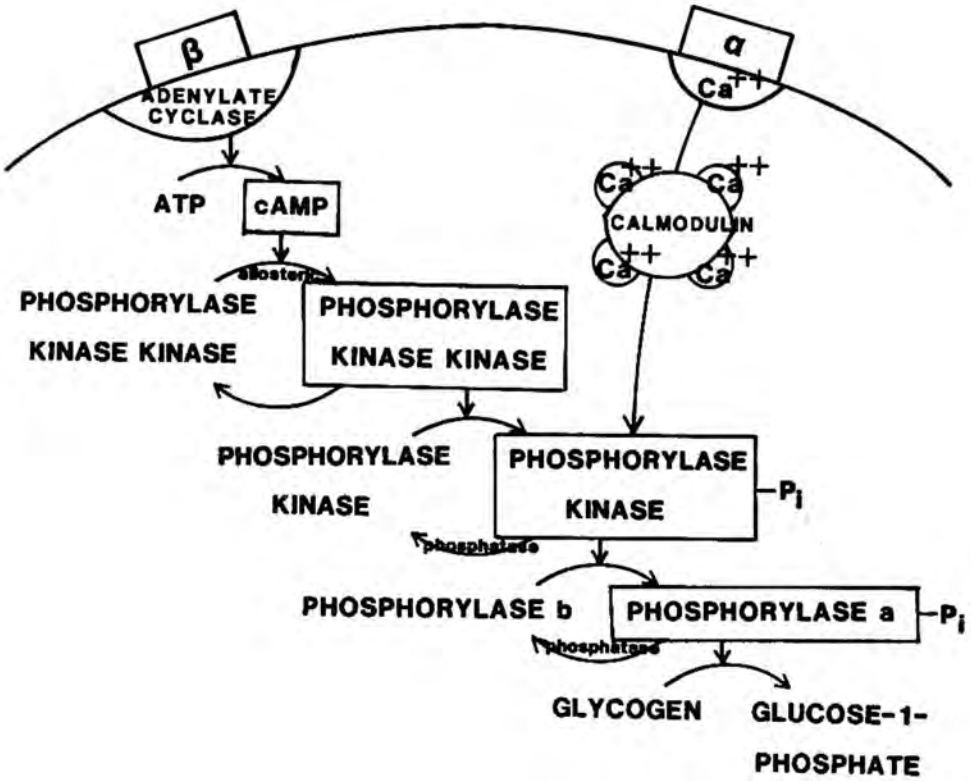
Biochemical Determination with Glycogen Phosphorylase a

Glycogen phosphorylase a activity is useful as an indirect measure of intracellular Ca^{++} concentrations. Phosphorylase activation in the absence of increases in cyclic AMP (cAMP) provides an "endogenous Ca^{++} indicator" in liver cells. Phosphorylase a activity is easily assayable and does not require disruptive cell loading techniques. Conversion of phosphorylase from the b to a form is mediated by glycogen phosphorylase kinase. While it is phosphorylase kinase that responds to increased Ca^{++} by autophosphorylation (Malencik and Fischer, 1982), appearance of phosphorylase a is more easily measured due to the glycogenolytic amplification cascade (Figure 3). The cascade for conversion of phosphorylase b to phosphorylase a can also be stimulated by cAMP (Exton et al., 1971). Thus, in order for elevated phosphorylase a activity to be attributed to increased Ca^{++}, it is essential that activation of phosphorylase kinase by cAMP be eliminated.

To allow reliable estimation of liver phosphorylase a levels in vivo, liver of anesthetized rats must be sampled in situ by a quick-freezing technique with liquid nitrogen-cooled aluminum tongs. These conditions minimize the production or destruction of tissue cAMP via local mechanisms or increased circulating catecholamines (Stahlmans et al., 1974). Phosphorylase a activity has been used successfully as a measure of cytosolic Ca^{++} concentrations in vivo with vasopressin

Figure 3. Cascade for Conversion of Glycogen Phosphorylase to the a Form

Glycogen phosphorylase is converted to the a form through phosphorylation by phosphorylase kinase. Phosphorylase kinase can be stimulated by increased intracellular Ca^{++} via calmodulin, or by phosphorylase kinase kinase (cAMP-dependent protein kinase) which is allosterically activated by cAMP.


β
ADENYLATE CYCLASE
α
Ca++
ATP
cAMP
Ca++
CALMODULIN
allosteric
PHOSPHORYLASE KINASE KINASE
PHOSPHORYLASE KINASE KINASE
PHOSPHORYLASE KINASE
PHOSPHORYLASE KINASE
Pi
phosphatase
PHOSPHORYLASE b
PHOSPHORYLASE a
Pi
phosphatase
GLYCOGEN
GLUCOSE-1-PHOSPHATE

(Keppens and deWulf, 1975) and angiotensin II (Keppens and deWulf, 1976). Phosphorylase activation has been determined in suspensions of rat hepatocytes with the same hormones (Keppens et al., 1977, Assimacopoulos-Jeannet et al., 1977). Burgess et al. (1983) reported that in guinea pig hepatocytes, phosphorylase a responds in a nearly linear fashion to logarithmic changes in Ca^{++} concentrations ranging from ten nM to ten uM. Murphy et al. (1980) demonstrated with rat hepatocytes that phosphorylase a activity increases within seconds and then decreases to basal activity within five minutes after stimulation by the alpha-agonist phenylephrine.

Fluorometric Determination With Quin2

Hepatic cytosolic Ca^{++} concentrations can be measured directly with the fluorescent Ca^{++} indicator, quin2. Tsien et al. (1982) developed this compound and used it to monitor intracellular Ca^{++} concentrations in mouse and pig lymphocytes. The acetoxymethyl ester of quin2 (quin2-AM) easily traverses cell membranes. Intracellular esterases then rapidly hydrolyze this form to yield free quin2, which is polar and remains trapped inside of cells. Quin2 has an EGTA-like structure; each molecule of dye binds one Ca^{++} and reports this interaction with increased fluorescence. Quin2 responds with logarithmically increased fluorescence over a range of Ca^{++} concentrations, from one nM to one uM. Calibration procedures with detergent and EGTA at the conclusion of each experiment allow calculation of intracellular Ca^{++} concentrations from fluorescence, independent of the level of dye loading (Tsien et al., 1982). Charest et al. (1983) and Berthon et al. (1984) successfully employed quin2 to monitor three- to five-fold increases in cytosolic Ca^{++} produced by the Ca^{++}-mobilizing hormones

norepinephrine and vasopressin in suspensions of rat hepatocytes. Binet et al. (1985) reported that quin2 responded within seconds after hormone addition to hepatocytes. However, these investigators also observed that maximally elevated quin2 fluorescence was maintained for five minutes or more after norepinephrine or vasopressin addition, exceeding the expected duration of action. This suggests that while quin2 rapidly binds Ca^{++} as concentrations increase, quin2 may not release Ca^{++} rapidly when concentrations fall. This potential limitation could become significant when contrasting the effects of hormones and toxic agents on cytosolic Ca^{++} concentrations.

HYPOTHESIS

Carbon Tetrachloride Elevates Cytoplasmic Calcium

CCl_4 can disrupt hepatic Ca^{++} homeostasis. In rats given a single hepatotoxic dose of CCl_4, ER Ca^{++} pump activity is severely inhibited within 30 minutes. ER Ca^{++} pump activity improves but does not fully recover within eight days following CCl_4 administration (Moore et al., 1976). Hepatic endoplasmic reticulum loses significant amounts of calcium in rats given CCl_4 (Reynolds et al., 1972, Moore, 1981, Kroner, 1982). These effects were observed in isolated membrane fractions derived from rats exposed to CCl_4. However, it was not known whether cytoplasmic Ca^{++} concentrations become elevated in intact liver cells as a result of disrupted ER Ca^{++} sequestration, or whether other Ca^{++} transporting mechanisms compensated by maintaining or restoring cellular Ca^{++} homeostasis. Recently, it was reported that the Ca^{++} pump found at plasma membranes is also inhibited by CCl_4 (Tsokos-Kuhn et al., 1985). Others have demonstrated that mitochondrial Ca^{++} transport is not affected at early times following CCl_4 administration (Carafoli and

Tiozzo, 1968, Moore et al., 1976).

The hypothesis that cytosolic Ca^{++} rises to supraphysiologic levels as a consequence of ER Ca^{++} pump inhibition was examined in liver from rats that received CCl_4 and in cultured hepatocytes exposed to CCl_4. CCl_4-induced alterations in the activities of phosphorylase a, ER Ca^{++} pump, and other enzymes were compared between these two preparations. In primary cultures of rat hepatocytes, the potential contribution of extracellular Ca^{++} to phosphorylase activation at early times was investigated. Biochemical (phosphorylase a) and fluorometric (quin2) methods for measurement of intracellular Ca^{++} concentrations were compared. The effects of CCl_4 on Ca^{++}-dependent enzyme activation were contrasted with the effects of phenylephrine, a non-hepatotoxin, hormone-like agent. The role of elevated cytosolic Ca^{++} in the development of CCl_4 hepatotoxicity was considered.

METHODS

IN VIVO STUDIES

Male, Sprague-Dawley rats weighing 200 to 250 g (Taconic Farms, Germantown, NY) were allowed one to two weeks to acclimatize to USUHS animal facilities before use. Rats received laboratory chow (Ralston-Purina, St. Louis, MO) and tap water ad lib., and were maintained on a 12 hour light/dark cycle. For in vivo studies, rats received intraperitoneal injections of CCl_4 (1.5 ml/kg, ACS grade, Kodak, Rochester, NY) or vehicle (corn oil) at 8:00 AM. Food was withheld from the animals after this time. Sampling procedures were initiated at 15, 30, 60, 120 minutes, 8 and 24 hours after CCl_4 administration. Fifteen minutes prior to the scheduled kill time, rats were given intraperitoneal injections of pentobarbital (50 mg/kg, Nembutal™, Abbott Labs., Chicago, IL). A midline incision was made on the abdomen of each anesthetized rat, and a portion of the left median lobe of the liver was rapidly frozen between liquid nitrogen-cooled aluminum tongs for phosphorylase and cAMP assays. A sample of blood was quickly taken by cardiac puncture for plasma enzyme determination. The diaphragm was then severed to sacrifice the rat. The remainder of the liver was excised and a two g portion was homogenized with 8 strokes at 750 rpm using a Potter-Elvejeham tissue grinder (Thomas Scientific, Philadelphia, PA) in ice-cold 0.25 M sucrose. A portion was mixed with an equal volume of 100 mM KCl and frozen in liquid nitrogen. Samples were stored at -70° C for subsequent enzyme determinations. Duplicate 0.5 g liver samples were also taken for total liver calcium determinations.

HEPATOCYTE PREPARATIONS

Isolation of Rat Hepatocytes

Male, Sprague-Dawley rats as described above were used for liver cell preparations. Prior to surgical procedures, each rat received an intraperitoneal injection of pentobarbital (50 mg/kg). Hepatocytes were prepared by a modification of the two-step, collagenase digestion method of Berry and Friend (1969). A midline abdominal incision was made, and the viscera were pushed aside. The renal blood supply was ligated. The inferior vena cava was isolated and cannulated with a 14 gauge stainless steel, blunt-tipped needle. Retrograde perfusion was immediately initiated with a HEPES-buffered salt and glucose solution (135 mM NaCl, 7 mM KCl, 12 mM glucose, 10 mM NaHEPES, pH 7.4) at 15 to 20 mls/minute. Simultaneously, the hepatic portal vein was severed and the superior vena cava was ligated. After 150 to 200 mls of the initial solution passed through the liver, the perfusion was immediately switched to 300 mls of the same solution supplemented with collagenase (90 units/ml, 130 to 160 units/g, Worthington Biochemicals, Freehold, NJ) and $CaCl_2$ (1 mM), adjusted to pH 7.5. Perfusion buffers were equilibrated with 100% oxygen and maintained at 42° C, so that they were 37° C as they entered the liver. The entire procedure required about half an hour. The liver was then removed from the rat. Cells were dispersed by gentle mechanical manipulation into 30 mls of Williams E medium (GIBCO, Grand Island, NY) supplemented with fetal bovine serum (10% by volume, Flow Labs., Rockville, MD), penicillin-streptomycin (50 units/ml, Flow Labs., Rockville, MD), glutamine (5 ug/ml, Flow Labs., Rockville, MD), a mixture of insulin (5 ug/ml), transferrin (5 ug/ml), and selenium (5 ng/ml) (ITS Premix, Collaborative Res., Lexington, MA), dexamethasone (4 ng/ml, Sigma Chem. Co., St. Louis, MO), and 10 mM NaHEPES (pH 7.4).

Liver cells were then filtered through nylon mesh (250 square microns, Tetko Corp., Elmsford, NY), and washed twice by low speed centrifugation (50 x g, two minutes) in order to remove dead (floating) cells. Final cell viability was assessed by counting the proportion of cells that excluded the dye trypan blue (Flow Labs., Rockville, MD) with a hemocytometer (American Optical, Buffalo, NY). Cell preparations with viabilities of less than 85% were discarded. Average viability was 93 $\pm$ 3% (mean $\pm$ SD). From the hemocytometer count, the yield of cells was determined to be 80 million cells per ml of loosely packed pellet formed in a 50 ml plastic, conical centrifuge tube after a two minute, 50 x g centrifugation. Excess pellet volume was aspirated to routinely yield 400 million cells from each liver.

Hepatocyte Cultures

Hepatocyte culture conditions were essentially those recommended by Bissell and Guzelian (1980). Cells were diluted to 0.6 million per ml in supplemented Williams E medium for plating onto 35 or 100 mm tissue culture dishes (Falcon™, Becton-Dickinson, Cockeysville, MD, or Lux™, Miles Labs., Naperville, IL). Dishes were pre-coated for one hour with a 40 ug/ml solution of collagen (Vitrogen™, Collaborative Res., Lexington, MA) in phosphate-buffered saline (150 mM NaCl, 2.5 mM KCl, 7.6 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4). Cells were seeded onto dishes at a density of 100,000 cells per square cm. Following a 45 minute attachment period, non-attached cells were aspirated and fresh medium was applied. Dishes were returned to a 5% CO_2 humidified tissue culture incubator overnight, to be used for experiments 18 to 24 hours later.

Hepatocyte Suspensions

Hepatocytes were prepared as freshly isolated suspensions for use in experiments with quin2. Cells were isolated as described above and suspended at 5 x 10^6 cells per ml in Krebs-Henseleit buffer containing 1 mM $CaCl_2$, 25 mM $NaHCO_3$, 93.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM HEPES, 10 mM glucose, pH 7.4, and supplemented with (final concentrations) 5 mM glutamate, 5 mM fumarate, 5 mM pyruvate, 2% bovine serum albumin (all from Sigma Chem. Co., St. Louis, MO), and Eagle's essential amino acids (Flow Labs., Rockville, MD). This medium was saturated with 100% oxygen before use. Hepatocyte suspensions were preincubated for 20 minutes in an orbital shaker at 100 cycles per minute, 37° C before use in experiments with quin2, described below.

Subcellular Fractionation

Four 150 mm dishes of cultured hepatocytes, or an equivalent volume of freshly isolated cells, were scraped into 2.5 mls of ice-cold 0.25 M sucrose with a teflon policeman. This yielded approximately six mls of a homogenate containing 10 to 15 mg protein/ml. Homogenates were disrupted by three bursts of sonication for ten seconds each (power level three, MicroUltrasonic Cell Disruptor, Kontes Glass Co., Vineland, NJ). Sonicates were then centrifuged at 1500 x g for ten minutes to pellet nuclei and unbroken cells. Supernatants were centrifuged at at 12,000 x g for 20 minutes to sediment mitochondria and cellular debris. The resulting supernatants were then centrifuged at 105,000 x g for 60 minutes to pellet microsomes and plasma membranes. ER Ca^{++} pump and glucose-6-phosphatase activities and protein content were quantitated in

all fractions. Cytochrome P-450 content and cytochrome c reductase activity were determined in microsomal fractions.

Addition of Halocarbons or Adrenergic Agents

CCl_4, bromotrichloromethane (both from Eastman Kodak, Rochester, NY), and chloroform (Mallinckrodt, St. Louis, MO) were dissolved in ethanol or dimethyl sulfoxide (as noted) and added with a gentle rocking motion to 35 mm tissue culture dishes of cells to be used for phosphorylase determinations. Dishes were maintained at 37° C on a slide warmer platform (Labline, Melrose Park, IL) for manipulations outside of the incubator. Solvent vapors were generated in selected experiments from liquid halocarbons (0.3 ml/L air) placed in a glass dish in a polycarbonate container (Nalge Co., Rochester, NY) fitted with an aluminum top and kept in a 37° C room. This chamber was opened in a chemical fume hood at the conclusion of each exposure period. All experiments were terminated by aspiration of the medium. Dishes with attached cells were immediately plunged into liquid nitrogen. Samples were stored at -70° C until they were assayed. Final concentrations of halocarbons ranged from 0.03 to 10 mM as indicated, and final maximum concentration of vehicle was 0.3% by volume. Isoproterenol and phenylephrine (both from Sigma Chem. Co., St. Louis, MO) exposures were performed by the same technique with water serving as vehicle. For quin2 determinations, CCl_4 (2 mM) or phenylephrine (10 uM) was added directly to the suspension of dye-loaded cells. Mixing was accomplished by rapid inversion of the fluorometer cuvette. The same vehicles were used as for phosphorylase a determinations.

PREPARATION OF CALIBRATION CURVE FOR PHOSPHORYLASE *a* AND CALCIUM

Cultured rat hepatocytes were permeabilized essentially as described by Burgess *et al*. (1983). Cells were exposed for ten minutes at 37° C to 50 ug/ml saponin (Sigma Chemical Co., St. Louis, MO) in a cytosol-like medium comprised of 115 mM KCl, 20 mM NaCl, 1.2 mM $MgCl_2$, 1 mM EGTA, and 10 mM HEPES, pH 7.1, as recommended by Tsien *et al*. (1982). Cells were subsequently exposed for ten minutes to a range of Ca^{++} concentrations appropriately buffered with EGTA (Sigma Chem. Co., St. Louis, MO) as described by Bers (1982). Phosphorylase *a* activity was then quantitated as described below. Free Ca^{++} concentrations achieved with Ca^{++}/EGTA buffers were calculated with the aid of microcomputer program written by S. Lee (provided by R. A. Steinhardt, Univ. of Calif., Berkeley, CA) using the Ca^{++}/EGTA dissociation constant of Martell and Smith (1974).

DETERMINATION OF CALCIUM WITH QUIN2

Quin2-AM (cell permeant acetoxymethyl ester form, Calbiochem, La Jolla, CA) was added to a final concentration of 50 uM to preincubated cell suspensions diluted to 2 x 10^6 cells per ml in Krebs-Henseleit medium without supplements. Hepatocytes were incubated with orbital shaking at 37° C for 5 minutes, the time required for hydrolysis of quin2-AM to free quin2. Cells were then collected by a five second centrifugation at 13,000 x g. Cells were resuspended in Krebs-Henseleit buffer at a final concentration of 10^6 cells per ml and transferred to a one cm square quartz cuvette. Fluorescence was monitored with a fluorometer (model MPF-44a, Perkin-Elmer Corp., Norwalk, CT) fitted with a stirring, 37° C water-jacketed cuvette holder. Excitation wavelength was 339 nm and emission wavelength was 500 nm; slit widths were five nm.

In order to calculate the Ca^{++} concentration from fluorescence (F), calibration procedures were performed as described by Tsien et al. (1982). Maximum quin2 fluorescence (F_{max}) for the dye loading was determined by addition of 20 uM digitonin (Sigma Chem. Co., St. Louis, MO), and minimum fluorescence (F_{min}) was obtained by addition of 4 mM EGTA (Sigma Chem. Co., St. Louis, MO). Corrections were made for changes in cell autofluorescence that occurred when these manipulations were performed with non-quin2-loaded hepatocytes. Ca^{++} concentrations were calculated as follows, assuming a dissociation constant (K_d) for Ca^{++}-quin2 of 115 nM:

$$[Ca^{++}] = K_d (F - F_{min}) / (F_{max} - F)$$

ENZYME ASSAYS

Glycogen phosphorylase a and a+b (Radiometric)

For in vivo studies, phosphorylase a and a+b activities were determined by the method of Golden et al. (1977) as incorporation of glucose from glucose-1-phosphate into glycogen. Liver tissue was disrupted by homogenization/sonication for ten seconds at maximum speed (Polytron, Brinkmann Inst., Westbury, NY) in a 30-fold volume of a buffer that prevented further phosphorylation or dephosphorylation of phosphorylase (100 mM NaF, 10 mM EDTA, 0.5% glycogen [Type II from oyster, Sigma Chem. Co., St. Louis, MO], 5 mM dithiothreitol, 50 mM Tris, pH 6.8). A 0.025 ml aliquot of this homogenate was added to 0.050 ml of phosphorylase a incubation buffer (150 mM NaF, 1.5% glycogen, 15 mM glucose-1-phosphate [Sigma Chem. Co., St. Louis, MO], 0.0015 mCi/ml [^{14}C]-glucose-1-phosphate [294 mCi/mmole, Amersham, Arlington Hts., IL], 0.75 mM caffeine, pH 6.2) or to 0.050 ml of phosphorylase a+b incubation buffer (same as for phosphorylase a, except caffeine was replaced with

7.5 mM AMP [Sigma Chem. Co., St. Louis, MO] and 10% 1,2-dimethoxyethane [Aldrich Chem. Co., Milwaukee, WI], which was added fresh on a daily [Uhing et al., 1979]). Samples were prepared as duplicates and incubated at 37° C for 12 minutes (phosphorylase a) or eight minutes (phosphorylase a+b). A 0.050 ml aliquot of the incubation mixture was applied to disposable ion-exchange columns made of siliconized pasteur pipettes containing a one-inch bed of AG1-X8 resin (acetate form, 100-200 mesh, Bio-Rad Labs., Richmond, CA). Columns retained the [^{14}C]-glucose-1-phosphate and allowed the newly synthesized [^{14}C]-glycogen to pass through. The column effluent was collected, including two 0.5 ml washes with water, and 10 mls of aqueous scintillation cocktail (EP, Beckman Inst., Fullerton, CA) were added. Samples were counted in a liquid scintillation counter (model 2450, Packard Tricarb, Downers Grove, IL). Assay conditions were linear with time and protein over the range that contained actual sample activities. Activities of phosphorylase a and a+b were expressed as nmoles of glucose incorporated into glycogen per mg protein per minute. In Figure 4, phosphorylase a activities were corrected for variations between rats in the total quantity of enzyme (a+b forms) present.

Glycogen phosphorylase a and a+b (Spectrophotometric)

For analysis of hepatocyte cultures, a spectrophotometric assay for phosphorylase a and a+b was devised that accomodated a reduced sample size and allowed assays to be performed directly in the 35 mm culture dishes. Assay conditions were modified from those recommended by Hue et al. (1975). The assay worked on the same principle as the radiometric assay, except that inorganic phosphate liberated from glucose-1-phosphate was quantitated in a colorimetric assay. Cells were

allowed to thaw in the presence of 0.25 ml of a lysing buffer (100 mM NaF, 20 mM EDTA, 0.5% glycogen, 50 mM glycyl-glycine, pH 7.4, and 0.5% Triton X-100) for five minutes on a rocker platform (Bellco, Vineland, NJ) in a 37° C room. The assay was started by addition of a 0.25 ml aliquot of phosphorylase a incubation buffer (100 mM glucose-1-phosphate, 2% glycogen, 1 mM caffeine, pH 6.1) or phosphorylase a+b incubation buffer (same as for phosphorylase a, except caffeine was replaced by 5 mM AMP and 10% dimethoxyethane [Uhing et al., 1979]). Dishes were covered to minimize evaporation and allowed to rock for 45 minutes at 37° C. Assays were ended by addition of one ml of ice-cold 5% trichloroacetic acid, and dishes were transferred to trays on ice for ten minutes to allow protein to precipitate. One ml samples were centrifuged for 30 seconds at 13,000 x g in 1.5 ml, plastic conical tubes in a microcentrifuge (model B, Beckman Inst., Fullerton, CA), and supernatants were immediately assayed for inorganic phosphate (method of Fiske and Subbarow [1925] modified to pH 2.5, see section on inorganic phosphate below). Pellets were digested in 0.1 ml of 1 N NaOH overnight at room temperature and retained for protein determination (see below). Samples were assayed under conditions that were linear with time and protein in the appropriate ranges. Activity of phosphorylase a was expressed as nmoles phosphate liberated per mg protein per minute. Good agreement was obtained between the spectrophotometric and radiometric assay procedures.

Endoplasmic Reticulum Calcium Pump

Endoplasmic reticulum (ER) Ca^{++} pump activity was assayed by the method of Moore et al. (1975) as ^{45}Ca accumulated by vesicles formed in a homogenate, under conditions optimized for Ca^{++} uptake by endoplasmic

reticulum. Liver homogenates were prepared as for the *in vivo* phosphorylase *a* assay and diluted to 0.2 to 0.4 mg protein/ml. Cell sonicates (five seconds at power level two, MicroUltrasonic Cell Disruptor, Kontes Glass Co., Vineland, NJ) were prepared in 0.125 M sucrose, 50 mM KCl to the same protein concentration. Duplicate 0.10 ml aliquots were added to tubes containing 0.90 ml of the ER Ca^{++} pump incubation mixture (100 mM KCl, 5 mM NaAzide, 5 mM NH_4Oxalate, 5 mM $MgCl_2$, 5 mM ATP, 0.020 mM $CaCl_2$, 0.001 mCi/ml $^{45}CaCl_2$ [18 mCi/mg, Amersham, Arlington Hts., IL], 30 mM imidazole, 30 mM histidine, pH 6.8) and incubated at 37° C for 30 minutes. Samples (0.5 ml) were applied to 25 mm, 0.45 micron filters (Millipore Corp., Bedford, MA), on a vacuum manifold (Amicon Corp., Danvers, MA). Filters were pre-rinsed with 0.25 M KCl and washed with 0.25 M sucrose following sample application. Filters were allowed to dry for 20 minutes under infrared heat lamps. Five mls of scintillation cocktail were added (NA, Beckman Inst., Fullerton, CA) and filters were counted in a liquid scintillation counter (model 2450, Packard Tricarb, Downers Grove, IL) to quantitate ^{45}Ca. Protein was determined (see below) and results were expressed as nmoles Ca^{++} pumped per mg protein in 30 minutes.

Mitochondrial Calcium Pump

Mitochondrial Ca^{++} pump activity was assayed essentially by the method of Carafoli (1967), with the exception that KCl was substituted for NaCl. Uptake of ^{45}Ca was determined in homogenates under conditions optimized for mitochondrial Ca^{++} sequestration. Cell sonicates were prepared in 0.125 M sucrose, 50 mM KCl as for the ER Ca^{++} pump. Duplicate 0.10 ml aliquots were added to tubes containing 0.90 ml of the mitochondrial Ca^{++} pump incubation mixture (10 mM Tris-HCl, pH 7.4, 10

mM $MgCl_2$, 80 mM KCl, 10 mM NaSuccinate, 3 mM ATP, 10 mM KH_2PO_4, 0.15 mM $CaCl_2$, 0.001 mCi/ml $^{45}CaCl_2$ [18 mCi/mg, Amersham, Arlington Hts., IL], and either 5 mM NaAzide or an equivalent volume of water) and incubated at 37° C for ten minutes. The assay was ended by application of 0.5 ml samples to 25 mm, 0.45 micron filters (Millipore Corp., Bedford, MA) on a vacuum manifold (Amicon Corp., Danvers, MA). Filters were pre-rinsed with 0.25 M KCl and washed with 0.25 M sucrose following sample application. Filters were allowed to dry for 20 minutes under infrared heat lamps, and five mls of scintillation cocktail were added (NA, Beckman Inst., Fullerton, CA). Samples were counted in a liquid scintillation counter (model 2450, Packard Tricarb, Downers Grove, IL) to quantitate ^{45}Ca accumulated. Protein was determined (see below) and results were expressed as nmoles Ca^{++} accumulated per mg protein in ten minutes. The difference between activities calculated from tubes with and without azide represented mitochondrial Ca^{++} pump activity.

Glucose-6-Phosphatase

Glucose-6-phosphatase was assayed by the method of Aronson and Touster (1974). Duplicate 0.050 ml aliquots of liver homogenates were added to tubes containing 0.45 ml of the assay mixture (20 mM NaGlucose-6-phosphate [Calbiochem-Behring, San Diego, CA], 1 mM EDTA, 17 mM imidazole, 17 mM histidine, pH 6.8) and incubated at 37° C for 15 minutes. Assays were ended by addition of 1.0 ml of ice-cold 5% trichloroacetic acid. Assay blanks consisted of incubations on ice, to which trichloroacetic acid addition was made prior to the addition of liver homogenate. Tubes were centrifuged at 1200 x g for 10 minutes, and inorganic phosphate liberated from glucose-6-phosphate was quantitated in the supernatant (method of Fiske and Subbarow [1925], see

section on inorganic phosphate below). Blank values were subtracted, and results were expressed as umoles phosphate liberated per mg protein in 15 minutes.

5′-Nucleotidase

5′-Nucleotidase was assayed by the method of Widnell and Unkeless (1968) using AMP as substrate. Duplicate 0.050 ml aliquots of liver homogenates were added to tubes containg 0.45 mls of the reaction mixture (100 mM AMP, 10 mM $MgCl_2$, 100 mM Tris, pH 8.5) and incubated at 37° C for 15 minutes. Assays were ended by addition of 1.0 ml of ice-cold 5% trichloroacetic acid. Assay blanks consisted of incubations on ice, to which trichloroacetic acid addition was made prior to the addition of homogenate. Tubes were centrifuged at 1200 x g for 10 minutes, and inorganic phosphate was quantitated in the supernatant (method of Fiske and Subbarow [1925], see section on inorganic phosphate below). Blank values were subtracted, and results were expressed as umoles phosphate liberated per mg protein in 15 minutes.

Glutamic-Pyruvic Transaminase

Glutamic-pyruvic transaminase activity in rat plasma, hepatocyte homogenates, and culture media was quantitated by the spectrophotometric method of Wroblewski and LaDue (1956) with a clinical test kit (SGPT-5, Sigma Chem. Co., St. Louis, MO). Disappearance of NADH was measured as decreasing absorbance at 430 nm with a recording spectrophotometer (model 250, Gilford Inst. Labs., Columbia, MD). Results for plasma were expressed as international units (IU) per ml, and for cells were expressed as [(100) x (IU in medium)] / [(IU in medium) + (IU in

hepatocyte homogenate)], which equals percent of intracellular enzyme activity found in the medium.

OTHER DETERMINATIONS

Inorganic Phosphate

Inorganic phosphate was determined following the glucose-6-phosphatase and 5′-nucleotidase assays by the colorimetric method of Fiske and Subbarow (1925). Aliquots (1.0 ml) were added to 3.5 mls of water and 0.5 ml of molybdate reagent (2.5% NH_4Molybdate, 5 $\underline{N}$ H_2SO_4). Two tenths ml of 1-amino-2-naphthol-4-sulfonic acid reagent (Fisher Scientific, Pittsburgh, PA) was added with vortexing, and samples were allowed five minutes for color development. Absorbance at 660 nm was measured with an auto-sampling spectrophotometer (Sargent-Welch, Skokie, IL). Color formation was linear from 0.1 to 1.0 micromole phosphate.

For inorganic phosphate determinations following the phosphorylase assays, a modified pH 2.5 (Lowry and Lopez, 1945) version of the above method was used. Compared to the pH 0.5 assay, there was less acid-catalyzed hydrolysis of the relatively labile glucose-1-phosphate, and a problem with precipitate formation was eliminated. All glassware (disposable and detergent-washed) had to be scrupulously avoided in order to prevent high blanks and erratic results, and therefore hand-rinsed or disposable plasticware was used without exception for phosphate determinations following phosphorylase assays. Aliquots (0.050 ml) of the trichloroacetic acid supernatants were added to 1.75 mls of glycine buffer pH 2.5, 0.45 ml of water, and 0.25 ml of modified molybdate reagent (2.5 % NH_4Molybdate, 0.05 $\underline{N}$ H_2SO_4). One tenth ml of 1-amino-2-napthol-4-sulfonic acid reagent was added with vortexing, and samples were allowed 30 minutes at 37° C for color development. Absor-

bance was measured at 660 nm. Color formation was linear from 0.05 to 0.5 umole phosphate.

Adenosine 3′:5′-Cyclic Monophosphate

Adenosine 3′:5′-cyclic monophosphate (cAMP) was measured by the protein binding displacement method of Gilman and Murad (1974) as modified by Brostrom and Kon (1974). Liver tissue was homogenized for ten seconds at maximum speed (Polytron, Brinkmann Inst., Westbury, NY) or hepatocytes were scraped with a teflon policeman into four volumes of ice-cold 5% trichloroacetic acid. Samples were centrifuged for ten minutes at 1200 x g. Supernatants were extracted twice with double volumes of water-saturated ether, and residual ether was dried off by heating to 60° C for 30 minutes. Duplicate 0.050 ml aliquots of extracts were added to tubes containing 0.050 ml of the cAMP incubation mixture (0.20 mg/ml protein kinase inhibitor [Sigma Chem. Co., St. Louis, MO], 0.1 uCi/ml [^{3}H]-cAMP [40 Ci/mmole, Amersham, Arlington Hts., IL], 50 mM $NaCH_3COO$, pH 4.5), to which 4.3 units of protein kinase (Sigma Chem. Co., St. Louis, MO) were added to start the assay. Tubes were incubated on ice for one hour, and then one ml of 20 mM K_2HPO_4, pH 6.0 was added. One ml samples were applied to 25 mm, 0.45 micron filters (Millipore Corp., Bedford, MA), on a vacuum manifold (Amicon Corp., Danvers, MA). Filters were pre-rinsed and washed after application of samples with 20 mM K_2HPO_4, pH 6.0. Filters were dried for 20 minutes under heat lamps, and then dissolved in one ml of ethyl acetate overnight. Ten mls of scintillation cocktail (NA, Beckman Inst., Fullerton, CA) were added, and samples were counted in a liquid scintillation counter (model LS 7800, Beckman Inst., Fullerton, CA). A plot of the logarithm of dpm vs. the logarithm of pmoles was linear from one

to 15 pmoles cAMP. Results were expressed as pmoles cAMP per mg protein.

Cytochrome P-450

Cytochrome P-450 content was measured by determining the amount of reduced, carbon monoxide-bound pigment from difference spectra, a modification of the method of Omura and Sato (1964). Microsomal fractions (0.5 to 0.7 mg protein/ml) were prepared from hepatocyte sonicates as described under Subcellular Fractionation, above. A 2.2 ml aliquot of the suspension was reduced by addition of approximately 1 mg $Na_2S_2O_4$, and the sample was divided between two cuvettes. A baseline spectrum was obtained with a double beam spectophotometer (AMINCO, American Inst. Co., Silver Spring, MD). One cuvette was then bubbled for ten seconds with carbon monoxide, and a difference spectrum was obtained. A microprocessor (MIDAN, American Inst. Co., Silver Spring, MD) aided in the computations of absorbance. Results were calculated from the absorbance difference at 450 nm and the difference extinction coefficient (91 cm^{-1} mM^{-1}), and expressed as nmoles cytochrome P-450 per mg microsomal protein.

Cytochrome c Reductase

Cytochrome c reductase activity was determined by the method of Phillips and Langdon (1962), by monitoring the rate of reduction of cytochrome c by NADPH. Microsomal fractions (0.5 to 0.7 mg protein/ml) were prepared from hepatocyte sonicates as described under Subcellular Fractionation, above. Two cuvettes were prepared, each with a 0.1 ml aliquot of the suspension added to 0.6 ml of reductase buffer (0.2 mM EDTA, 0.6 M K_2HPO_4/KH_2PO_4, pH 7.7), 0.1 ml of cytochrome c in solution

(5.13 mg/ml in water, Sigma Chem. Co., St. Louis, MO), and 0.3 ml of water. A baseline was recorded with a double beam spectophotometer (AMINCO, American Inst. Co., Silver Spring, MD). The assay was started by addition of 0.1 ml of NADPH (1.04 mg/ml in water, prepared fresh daily, Sigma Chem. Co., St. Louis, MO) to one cuvette. Increasing absorbance at 550 nm was recorded with time. Results were calculated from the change in absorbance at 550 nm per minute and the extinction coefficient for cytochrome c (21 cm^{-1} mM^{-1}), and expressed as nmoles cytochrome c reduced per minute.

Total Liver Calcium

Total liver calcium (ionized and complexed) was determined by atomic absorption spectrophotometry as described by Schmidt and Way (1979). Liver samples (0.5 g) were dried in porcelain crucibles at 95° C overnight, and then ashed at 600° C for 2 days. Ashed livers were resuspended in 1% $LaCl_2$ in 0.1 N HCl, and calcium was determined with a atomic absorption spectrophotometer (model 603, Perkin-Elmer Corp., Norwalk, CT). Absorbance was linear from 0.1 to 10 ug calcium/ml. Results were expressed as ug calcium per g liver.

Glycogen

Glycogen was recovered by modification of the ethanol precipitation method of Good et al. (1933). Duplicate 0.05 g samples of liver tissue were heated to 99° C in 20 volumes of 27% KOH until dissolved. One tenth ml of 10% Na_2SO_4 and 4.4 mls of 95% ethanol were added, and glycogen was allowed to precipitate overnight. Samples were centrifuged at 2500 x g for 15 minutes. The recovered pellet was washed with two volumes of 66% ethanol, recentrifuged, and then heated to 60° C for 30

minutes to remove remaining ethanol. Glycogen was enzymatically hydrolyzed to glucose residues by the method of Huijing (1970). Pellets were resuspended in two mls of 100 mM $NaCH_3COO$, pH 4.8, containing 0.50 unit amyloglucosidase (from Aspergillus niger, Boehringer Mannheim, Indianapolis, IN), and 0.38 units of alpha-amylase (from porcine pancreas, Sigma Chem. Co., St. Louis, MO). Samples were incubated for two hours at 30° C. Glucose was then measured by a modification of the method of Raabo and Terkisen (1960). Duplicate aliquots of the sample (0.010 ml) were made up to 1.0 ml with 100 mM K_2HPO_4, pH 6.5. One ml was added of a freshly prepared glucose reagent solution containing 12.5 units glucose oxidase (from Aspergillus niger, Sigma Chem. Co., St. Louis, MO), 2.5 units horseradish peroxidase (Sigma Chem. Co., St. Louis MO), and 0.1 mg o-dianisidine (Sigma Chem. Co., St. Louis, MO) in 100 mM K_2HPO_4, pH 6.5. Samples were incubated at 37° C for 20 minutes, and then absorbance was measured at 450 nM. The assay was linear for the detection of 1 to 30 ug glucose. Results were expressed as mg glucose liberated from glycogen per g liver.

Protein

Protein was quantitated by colorimetric assays, using bovine serum albumin (Fraction V, Sigma Chem. Co., St. Louis, MO) as a standard. For in vivo studies, protein was determined by the method of Bradford (1976) with a Bio-Rad assay kit (Bio-Rad Labs., Richmond, CA). For studies with hepatocytes, protein was quantitated by a modification of the method of Lowry et al. (1951), as was described by Shatkin (1969). Assays were linear for the detection of 5 to 25 ug protein.

STATISTICS

Statistical differences were detected by Student's t-test, or one- or two-way analysis of variance (ANOVA), as appropriate (Armitage, 1971). ANOVAs were calculated with the use of SAS (Statistical Analysis System, SAS Institute, Inc., Cary NC). N represented the number of animals or hepatocyte preparations used for an experiment. Significant differences after application of ANOVAs were determined by least significant difference test of the F-values (Winer, 1971). Significance for all tests was set at the p equals 0.05 level.

RESULTS

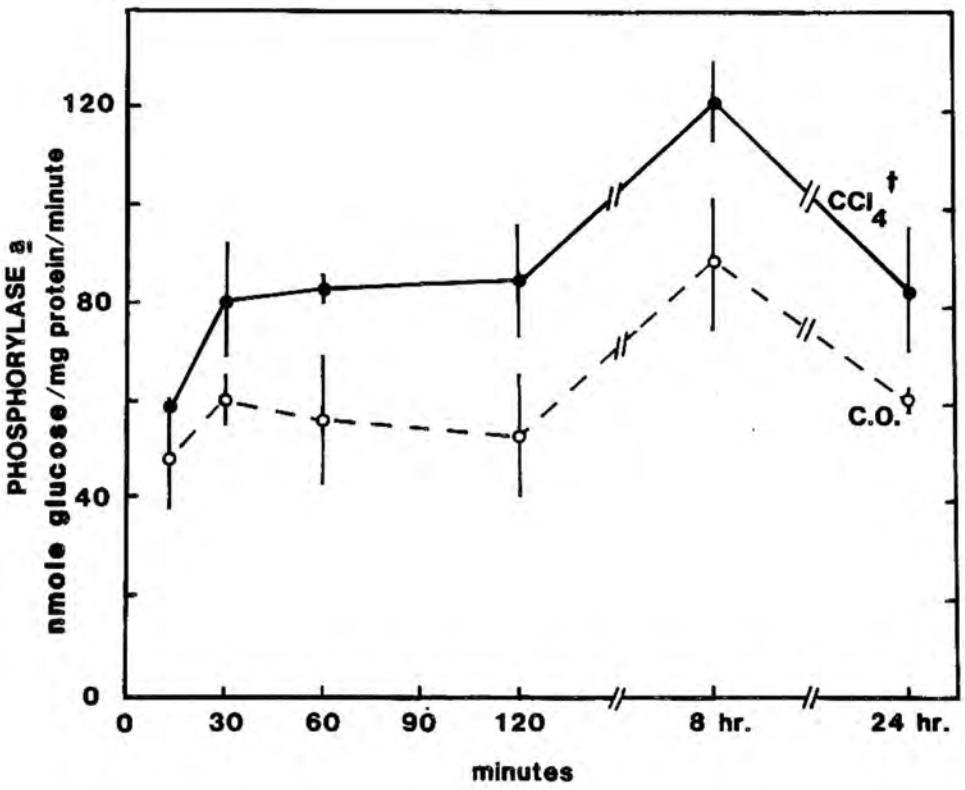
EFFECTS OF CARBON TETRACHLORIDE ADMINISTERED IN VIVO

Phosphorylase a, Phosphorylase a+b, and Glycogen

Time course studies were performed over 24 hours with rats administered a single hepatotoxic dose of CCl_4 (1.5 ml/kg). Glycogen phosphorylase a is known to respond to animal stress and diurnal rhythms (Newman and Armstrong, 1978, Theen et al., 1982), therefore sample collection was performed under carefully controlled experimental conditions. Time of day, feeding schedule, animal anesthetization, and tissue collection via rapid-freezing techniques were arranged (see Methods) to minimize artifactual changes in the level of phosphorylase a activity. When basal phosphorylase a activity was examined in liver from control animals (Figure 4), variations as great as two-fold (48 to 86 nmole/mg protein/minute) were observed. Maximal basal phosphorylase a activity was observed at the eight hour time point, which occurred when the animals were quiet and not eating (4:00 PM). Hepatic phosphorylase a activity fluctuates diurnally in order to maintain blood glucose levels through mobilization of liver glycogen (Ishikawa et al., 1976). In liver from rats that received CCl_4, phosphorylase a activity was 33% higher (81 vs. 60 nmole/mg protein/minute) as early as 30 minutes after CCl_4, and 50% higher (84 vs. 56 nmole/mg protein/minute) by 60 minutes after CCl_4 (Figure 4). Normal diurnal fluctuations were observed in liver from both CCl_4-treated and control rats. At 24 hours, hepatic phosphorylase a levels remained elevated in rats that received CCl_4. The difference between phosphorylase a activity measured in CCl_4-treated and control animals was significant for the treatment as a whole. In order to establish that the increase in phosphorylase a activity after

Figure 4. Liver Phosphorylase *a* Activity After Carbon Tetrachloride *In Vivo*

Carbon tetrachloride (1.5 ml/kg) or corn oil was administered at zero minutes. Rats were killed at 30 minutes through 24 hours, and samples of liver tissue were collected with aluminum tongs cooled in liquid nitrogen. Phosphorylase *a* activity was quantitated as nmoles ^{14}C-glucose incorporated into glycogen/mg protein/minute. Data represent means $\pm$ SEM from three to five rats at each timepoint. Differences between treatments as a whole are significant at $p \leq 0.05$ (+) by F-test following two-way ANOVA.


PHOSPHORYLASE a
nmole glucose/mg protein/minute
120
80
40
0
0
30
60
90
120
8 hr.
24 hr.
minutes
CCl4 †
C.O.

CCl_4 did not represent a change in the total quantity of enzyme present, total liver phosphorylase activity (a and b forms) was determined (Table 1). For the first eight hours, no significant differences were found between animals that received CCl_4 or those that received corn oil. Phosphorylase a+b activity ranged from 162 to 209 nmole/mg protein/minute. At 24 hours, significantly less total phosphorylase activity was present in the liver of rats that received CCl_4. This probably reflected the more generalized cellular damage that had occurred by this time. Changes in glycogen content reflected the net balance of the opposing activities of activated glycogen phosphorylase and activated glycogen synthetase (Hems and Whitton, 1980), thus glycogen levels were quantitated in liver from rats that received CCl_4 or corn oil (Figure 5). In control animals, a decline in glycogen content from 40 mg/g liver to essentially zero by 24 hours was observed. This occurred when food was withheld from all animals after dosing, because CCl_4-treated rats do not eat. In rats given CCl_4, liver glycogen content was 33% lower (28 vs. 42 mg/g liver) at 30 minutes. It remained lower than in control rats until eight hours after CCl_4, when liver glycogen was completely depleted. Up to eight hours, glycogen reserves declined at essentially the same rate in all rats. Paradoxically, glycogen content was increased slightly at 24 hours after CCl_4. Apparently some glycogen synthesis was occurring in the liver of rats recovering from CCl_4. The stomachs of these rats contained food at this time, unlike the stomachs of control rats (food was withheld from all animals after dosing). The difference in liver glycogen content between control and CCl_4-treated rats was significant for the treatment as a whole. Overall lower liver glycogen concentrations after CCl_4 resulted from prolonged activation of

Table 1. Liver Phosphorylase a+b Activity After Carbon Tetrachloride In Vivo

Carbon tetrachloride (1.5 ml/kg) or corn oil was administered at zero minutes. Rats were killed at 30 minutes through 24 hours, and samples of liver tissue were collected with aluminum tongs cooled in liquid nitrogen. Total phosphorylase activity was quantitated as nmoles ^{14}C-glucose from glucose-1-phosphate incorporated into glycogen/mg protein/minute, under conditions that allowed both a and b forms of phosphorylase to be active. Data represent means ± SEM from three to five rats at each timepoint. Differences between treatments at each timepoint are significant at $p \leq 0.05$ (*) by Student's t-test.

TABLE 1.

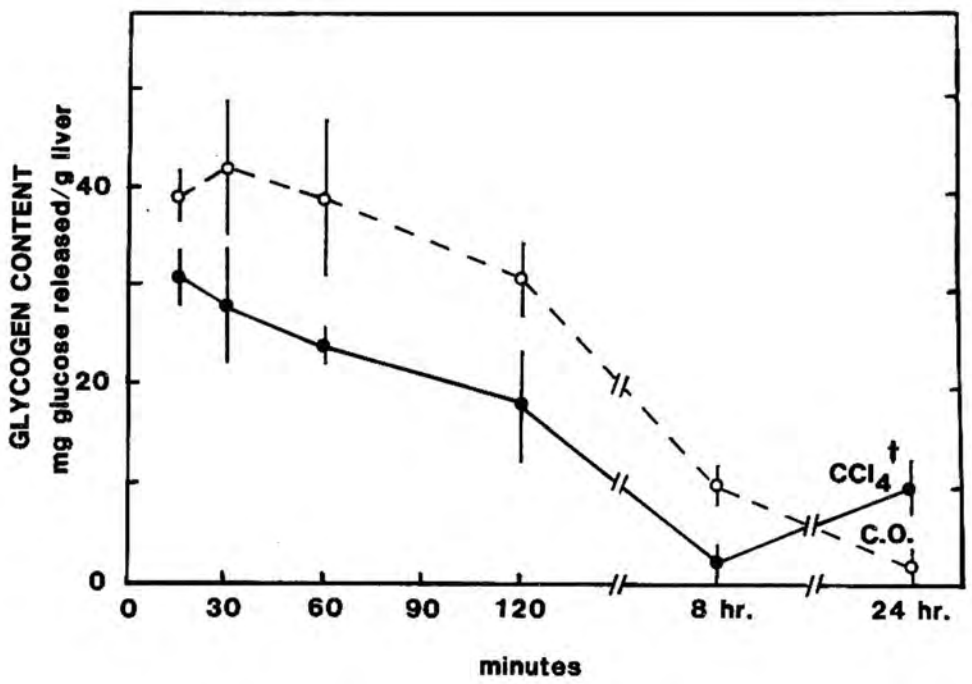
PHOSPHORYLASE a + b ACTIVITY IN VIVO

(nmole/mg protein/minute)

	15 min.	30 min.	60 min.	120 min.	8 hr.	24 hr.
Corn Oil	162 ± 15	166 ± 8	163 ± 6	178 ± 8	194 ± 8	172 ± 8
CCl_4	175 ± 10	178 ± 12	177 ± 7	177 ± 9	209 ± 10	131 ± 6*

Figure 5. Liver Glycogen Content After Carbon Tetrachloride *In Vivo*

Carbon tetrachloride (1.5 ml/kg) or corn oil was administered at zero minutes. Rats were killed at 30 minutes through 24 hours, and samples of liver tissue were collected with aluminum tongs cooled in liquid nitrogen. Glycogen content was quantitated as mg glucose liberated from ethanol-precipitated glycogen/g liver. Data represent means $\pm$ SEM from three to five rats at each timepoint. Differences between treatments as a whole are significant at $p \leq 0.05$ (+) by F-test following two-way ANOVA.


GLYCOGEN CONTENT
mg glucose released/g liver
40
20
0
0
30
60
90
120
8 hr.
24 hr.
minutes
CCl4
C.O.

phosphorylase and thus corroborated the observation that phosphorylase a levels were elevated. An additional or alternative explanation is that glycogen synthetase was inactivated following CCl_4; normally glycogen phosphorylase and glycogen synthetase are reciprocally regulated enzymes (Hems and Whitton, 1980).

Endoplasmic Reticulum Calcium Pump

ER Ca^{++} pump activity in liver from control rats averaged 175 nmole Ca^{++}/mg protein/30 minutes and did not vary significantly throughout the 24 hour duration of the experiment (Figure 6). Following CCl_4 administration, ER Ca^{++} pump activity was significantly depressed to 49 nmole/mg protein/30 minutes by 30 minutes, and maximally depressed to 36 nmole/mg protein/30 minute by 60 minutes. Pump activity did not recover by 24 hours later. Inhibition was significant at 30 minutes and all times thereafter. These findings reproduced the rapid and severe inhibition of the ER Ca^{++} pump that had been reported by others (Moore et al., 1976, Lowrey et al., 1981).

Glucose-6-Phosphatase and 5'-Nucleotidase

In order to determine whether inhibition of the ER Ca^{++} pump caused by CCl_4 was relatively selective, the activity of another enzyme located at hepatic endoplasmic reticulum, glucose-6-phosphatase, was quantitated (Figure 7). In control animals, glucose-6-phosphatase activity averaged 1.59 umole/mg protein/20 minutes and did not vary significantly throughout the course of the experiment. In rats given CCl_4, this enzyme was not significantly affected until 120 minutes, when glucose-6-phosphatase was inhibited to 1.21 umole/mg protein/20 minutes, and by eight and 24 hours after CCl_4 when the activity of this enzyme

Figure 6. Liver Endoplasmic Reticulum Calcium Pump Activity After Carbon Tetrachloride *In Vivo*

Carbon tetrachloride (1.5 ml/kg) or corn oil was administered at zero minutes. Rats were killed at 30 minutes through 24 hours, and liver homogenates were prepared. Calcium pump activity was quantitated as nmoles ^{45}Ca accumulated by vesicles formed in liver homogenate/mg protein/30 minutes. Data represent means $\pm$ SEM from three to five rats at each timepoint. Differences between treatments at each timepoint are significant at $p \leq 0.05$ (*) by Student's t-test.

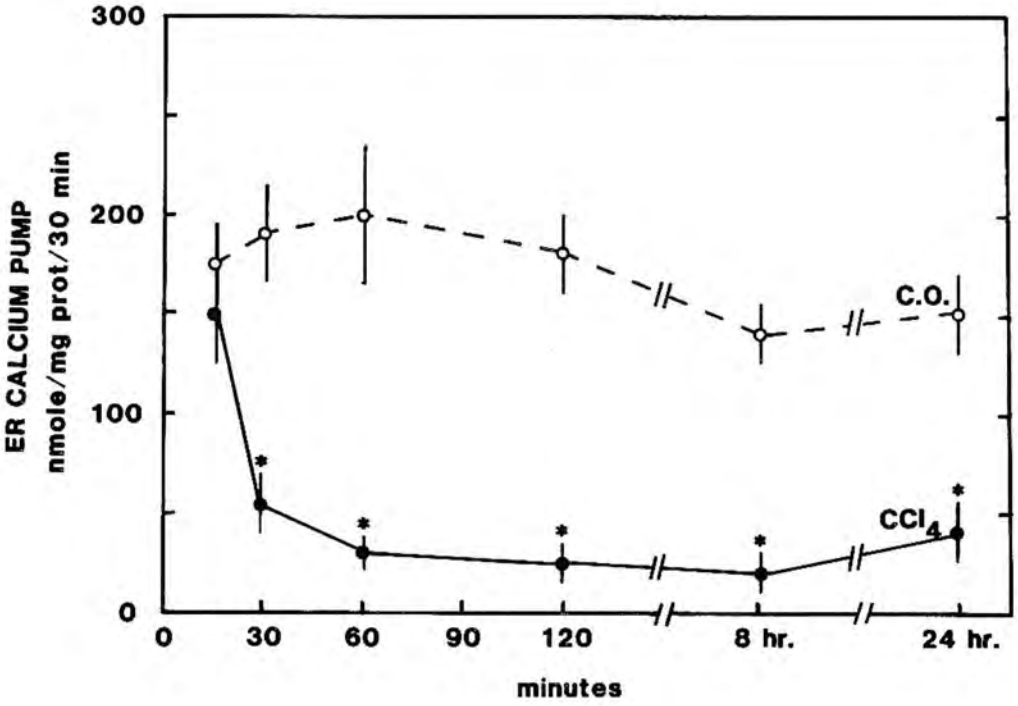
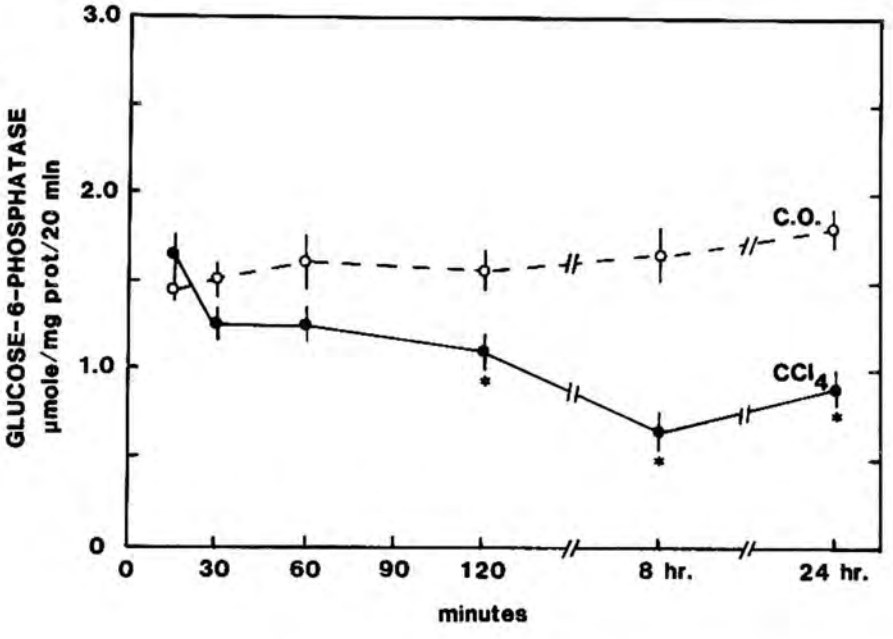

300
200
100
0
ER CALCIUM PUMP
nmole/mg prot/30 min
C.O.
CCl4
*
0
30
60
90
120
8 hr.
24 hr.
minutes

Figure 7. Liver Glucose-6-Phosphatase Activity After Carbon Tetrachloride *In Vivo*

Carbon tetrachloride (1.5 ml/kg) or corn oil was administered at zero minutes. Rats were killed at 30 minutes through 24 hours, and liver homogenates were prepared. Glucose-6-phosphatase activity was quantitated as umoles inorganic phosphate released from glucose-6-phosphate/mg protein/20 minutes. Data represent means $\pm$ SEM from three to five rats at each timepoint. Differences between treatments at each timepoint are significant at $p \leq 0.05$ (*) by Student's t-test.


GLUCOSE-6-PHOSPHATASE
µmole/mg prot/20 min
3.0
2.0
1.0
0
C.O.
CCl4
*
*
*
0
30
60
90
120
8 hr.
24 hr.
minutes

was approximately halved. Results obtained for glucose-6-phosphatase inhibition essentially duplicated that found by other investigators (Klaassen and Plaa, 1969). Glucose-6-phosphatase was not affected as early as the ER Ca^{++} pump, and never as severely. In order to assess the integrity of hepatic plasma membranes, the activity of an enzyme located there, 5′-nucleotidase, was determined (Figure 8). In liver from both control and CCl_4-treated rats, 5′-nucleotidase activity averaged 0.98 umole/mg protein/20 minutes. No significant changes were observed throughout the duration of the experiment.

Glutamic-Pyruvic Transaminase and Total Liver Calcium

Two indices of overt liver toxicity were examined in rats that received CCl_4. Blood levels of glutamic-pyruvic transaminase, a liver cytosolic enzyme, were determined as an indicator of hepatic damage (Wroblewski and LaDue, 1956). In serum from control animals, glutamic-pyruvic transaminse activity was low (20 IU/liter) and did not vary throughout the course of the experiment (Figure 9). At eight and 24 hours after CCl_4 administration, serum enzyme levels were 9-fold and 11-fold control, respectively, and these differences were significant. Accumulation of total liver calcium (ionized and complexed) has been found experimentally to accompany tissue necrosis (reviewed by Trump et al., 1982). Total liver calcium was 50 ug/g liver in liver from control rats at all times. Liver calcium rose to 2.2-fold and 4.4-fold control levels at eight and 24 hours after CCl_4, respectively (Figure 10), and these increases were statistically significant. Serum transaminase determinations and total liver calcium levels essentially duplicated that previously reported by others (Reynolds, 1963, Reynolds, 1964). These results demonstrated that the dose of CCl_4 used did not produce

Figure 8. Liver 5′-Nucleotidase Activity After Carbon Tetrachloride *In Vivo*

Carbon tetrachloride (1.5 ml/kg) or corn oil was administered at zero minutes. Rats were killed at 30 minutes through 24 hours, and liver homogenates were prepared. 5′-Nucleotidase activity was quantitated as umoles inorganic phosphate released from adenosine monophosphate/mg protein/20 minutes. Data represent means ± SEM from three to five rats at each timepoint. Differences between treatments at each timepoint are not significant by Student′s t-test.

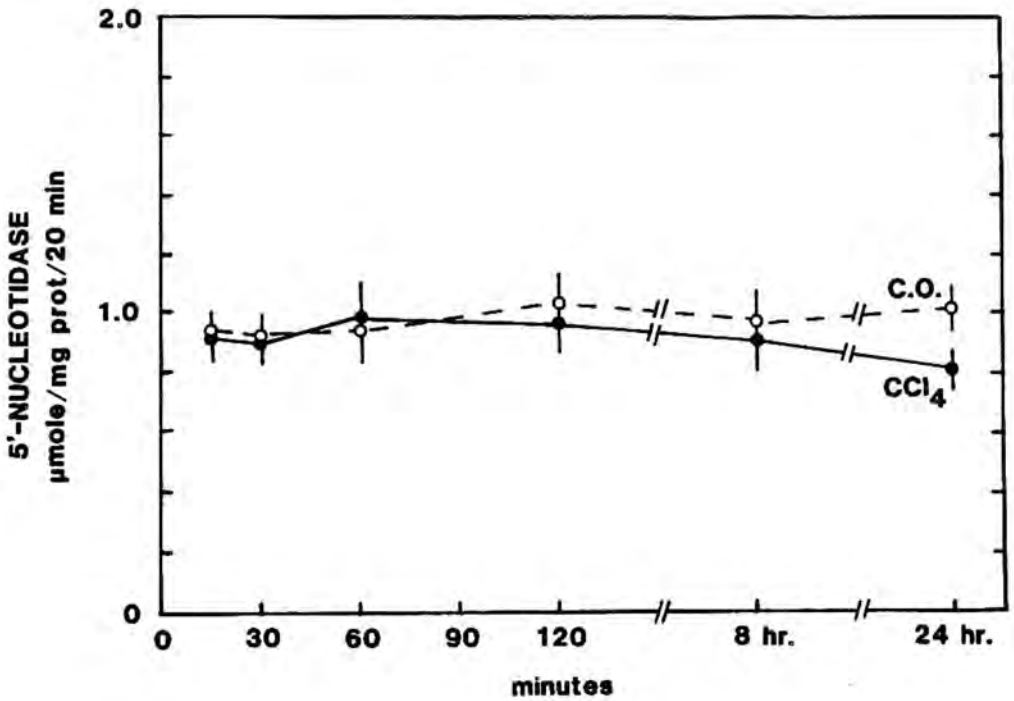

2.0
1.0
0
5'-NUCLEOTIDASE
µmole/mg prot/20 min
C.O.
CCl4
0
30
60
90
120
8 hr.
24 hr.
minutes

Figure 9. Serum Glutamic-Pyruvic Transaminase Activity After Carbon Tetrachloride *In Vivo*

Carbon tetrachloride (1.5 ml/kg) or corn oil was administered at zero minutes. Rats were killed at 30 minutes through 24 hours and blood was collected. Glutamic-pyruvic transaminase activity in serum was quantitated by monitoring oxidation of NADPH spectrophotometrically, and expressed as international units/liter. Data represent means $\pm$ SEM from three to five rats at each timepoint. Differences between treatments at each timepoint are significant at $p \leq 0.05$ (*) by Student's t-test.

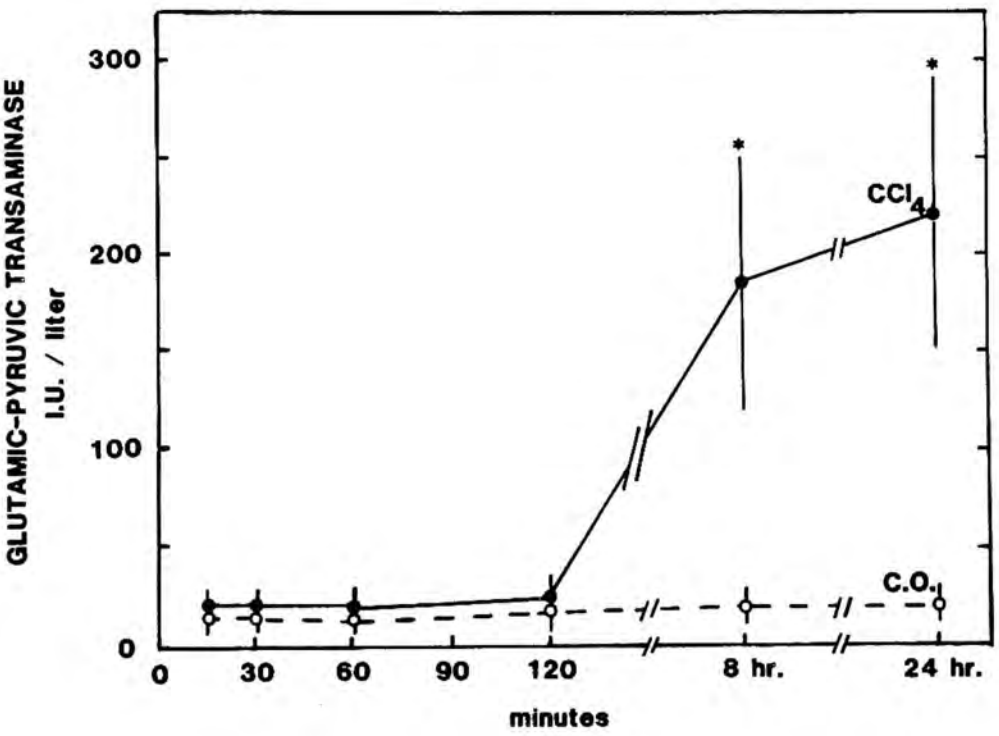
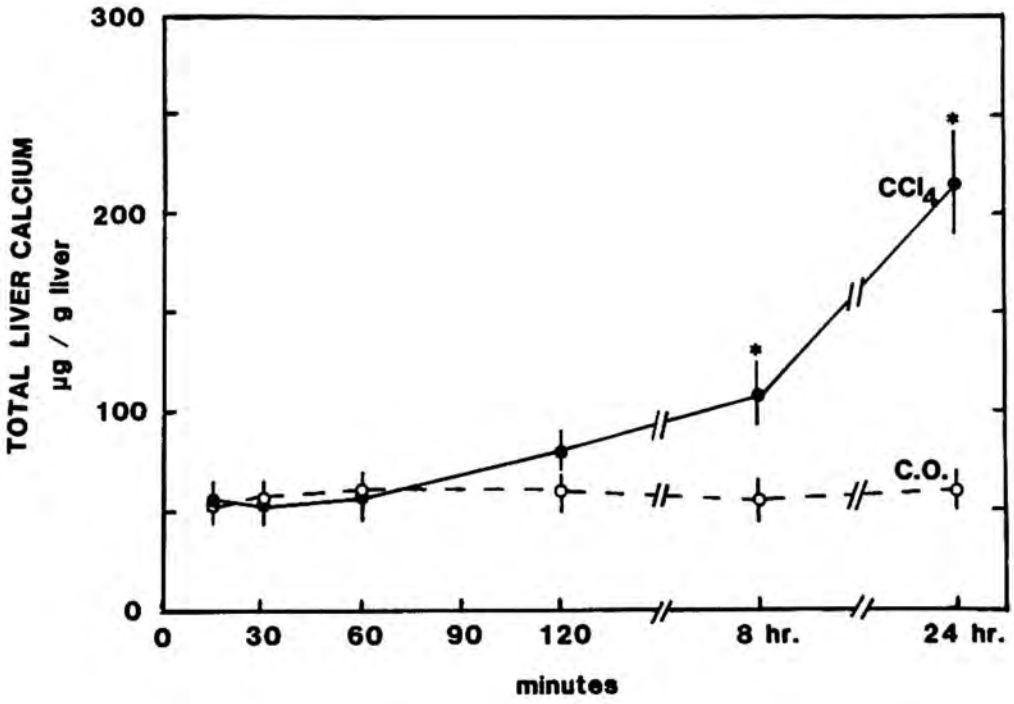

GLUTAMIC-PYRUVIC TRANSAMINASE
I.U. / liter
300
200
100
0
CCl4
C.O.
0
30
60
90
120
8 hr.
24 hr.
minutes

Figure 10. Liver Total Calcium After Carbon Tetrachloride *In Vivo*

Carbon tetrachloride (1.5 ml/kg) or corn oil was administered at zero minutes. Rats were killed at 30 minutes through 24 hours, and ashed liver samples were prepared. Total calcium content was quantitated by atomic absorption spectrophotometry and expressed as ug calcium/g liver. Data represent means $\pm$ SEM from three to five rats at each timepoint. Differences between treatments at each timepoint are significant at $p \leq 0.05$ (*) by Student's t-test.


TOTAL LIVER CALCIUM
µg / g liver
300
200
100
0
CCl4
C.O.
*
*
0
30
60
90
120
8 hr.
24 hr.
minutes

evidence of severe liver injury within the first two hours, although substantial tissue necrosis certainly occurred by eight to 24 hours later. These late hepatotoxic events may have resulted from early disruption of cell metabolic functions caused by disturbed intracellular Ca^{++} homeostasis that was apparent as early as 30 minutes after CCl_4 administration.

Adenosine 3′:5′-Cyclic Monophosphate

Levels of phosphorylase a can increase as a result of intracellular generation of cAMP (Exton et al., 1971). In order to determine whether this alternative mechanism contributed to the observed increase in phosphorylase a activity after CCl_4, cAMP content of liver tissue was quantitated (Table 2). In liver from control rats, cAMP concentration averaged 0.7 pmoles/mg liver, in good agreement with results obtained for this tissue by other investigators (MacManus et al., 1972). In liver from animals that received CCl_4, no measurable increases in cAMP levels were found by 120 minutes after CCl_4, a slight but non-significant increase was observed at eight hours, and no difference was found at 24 hours. Thus, within the first two hours after CCl_4 administration, elevated phosphorylase a activity was not accompanied by detectable increases in cAMP. These results were consistent with the interpretation that the elevation in liver phosphorylase a after CCl_4 administration in vivo was Ca^{++}-dependent.

CHARACTERIZATION OF RAT HEPATOCYTE CULTURES

An in vitro model was needed for further experimentation to discern the role of Ca^{++} in elevated liver phosphorylase a activity independent of other physiological influences on hepatic function (e.g.

Table 2. Cyclic AMP Content of Liver After Carbon Tetrachloride *In Vivo*

Carbon tetrachloride (1.5 ml/kg) or corn oil was administered at zero minutes. Rats were killed at 30 minutes through 24 hours, and samples of liver tissue were collected with aluminum tongs cooled in liquid nitrogen. Cyclic AMP was determined by displacement of ^{3}H-cAMP in a protein binding assay as described in Methods. Results were expressed as pmoles cAMP/mg liver. Data represent means ± SEM from three to five rats at each timepoint. Differences between treatments at each time-point are not significant by Student's t-test.

TABLE 2.

LIVER CYCLIC AMP CONTENT *IN VIVO*

(pmole/mg liver)

	15 min.	30 min.	60 min.	120 min.	8 hr.	24 hr.
Corn Oil	.59±.09	.61±.06	.60±.10	.55±.08	.69±.10	.76±.03
CCl_4	.71±.04	.70±.05	.60±.04	.63±.05	.99±.12	.79±.01

endocrine, neural, and cardiovascular systems). In vitro experiments avoid difficulties encountered in in vivo experiments with diurnal fluctuations in basal phosphorylase activities. Isolated hepatocytes can be prepared and maintained under tightly controlled, easily manipulated experimental conditions. Hepatocyte preparations are used for toxicity studies either as fresh suspensions or as primary cultures. Primary cultures of rat hepatocytes (quiescent, non-dividing cells) were selected as a model, because these cells have recovered from the enzymatic and mechanical rigors of the collagenase perfusion and isolation procedures. Cultured hepatocytes have replenished ATP stores, hormonal responsiveness, and metabolic functions superior to that found in freshly isolated cell suspensions (Bissell et al., 1973). Established techniques for the preparation of rat hepatocyte cultures were adopted for use in our laboratory. These cultures consisted mainly of liver parenchymal cells that were maintained for 18 to 24 hours after isolation. Composition of the hormone and trace mineral supplemented medium was selected in order to minimize loss of cytochrome P-450 content that occurs when hepatocytes are maintained in culture (Bissell and Guzelian, 1980). When viewed by phase contrast light microscopy, hepatocytes in culture appeared tightly attached and flattened. Most cells were in direct contact with other cells on all sides. Membrane protrusions (blebs) observed in freshly isolated cells were repaired after 18 hours, suggesting that plasma membrane integrity had improved with time in culture (R. M. Long, unpublished). This was particularly important because others have cautioned that CCl_4 exposure can directly damage the plasma membrane of freshly isolated hepatocytes (Perrissoud, et al., 1981, Stacey and Fanning, 1981).

Enzyme Distribution after Subcellular Fractionation

Subcellular fractions were prepared from fresh and cultured hepatocytes, and the distribution of marker enzymes was evaluated. The eventual goal was comparison between liver preparations of microsomal cytochrome P-450 content and cytochrome c reductase activity (both enzymes are essential for mixed function oxidase activity). Cells were broken by sonication and subjected to differential centrifugation (see Methods). Pellets were collected that contained mainly nuclei and unbroken cells, mitochondria and associated debris, microsomes and plasma membranes, along with high speed supernatants. Distribution of protein, glucose-6-phosphatase, and ER Ca^{++} pump activities in fractions from cultured hepatocytes, freshly isolated hepatocytes, and rat liver are compared in Tables 3, 4, and 5. The proportion of recovered protein found sedimented in the microsomal fraction from cultured cells, fresh cells, and liver homogenate was 14.4%, 25.0%, and 20.7%, respectively. The proportion of glucose-6-phosphatase activity recovered in the microsomal fraction from cultured cells, fresh cells, and liver homogenate was 28.3%, 42.0%, and 52.5%, respectively. The proportion of ER Ca^{++} pump activity found in the microsomal fraction from cultured cells, fresh cells, and liver homogenate was 43.7%, 61.0%, and 77.7%, respectively. Thus, distribution upon fractionation of recovered protein and enzymes of the endoplasmic reticulum was comparable between freshly isolated cells and liver homogenate, but lesser quantities were found in the microsomal fraction from cultured cells. A 2.3- and 1.4-fold enrichment of the specific activities of glucose-6-phosphatase and ER

Table 3. Distribution of Protein, Glucose-6-Phosphatase, and Endoplasmic Reticulum Calcium Pump Activities in Fractions from Cultured Hepatocytes

Sonicates of cultured hepatocytes were subjected to centrifugation at 1500 x g for ten minutes, 12,000 x g for 20 minutes, and 105,000 x g for one hour to sediment pellets enriched in nuclei, mitochondria, and microsomes, respectively. Protein and enzyme activities were determined in original homogenates and in all fractions, as described in Methods. Data represent means $\pm$ SEM from three cell preparations. Percent recoveries after fractionation were as follows: protein, 88.0 $\pm$ 12%; glucose-6-phosphatase, 110 $\pm$ 18%; endoplasmic reticulum calcium pump, 57.0 $\pm$ 6.6%.

TABLE 3.

DISTRIBUTION OF PROTEIN, GLUCOSE-6-PHOSPHATASE, AND ER CALCIUM PUMP ACTIVITIES IN FRACTIONS FROM CULTURED HEPATOCYTES

Fraction	Distribution of Recovered Protein (%)	G-6-Pase Activity (umol/mg prot/20′)	Distribution of Recovered G-6-Pase Activity (%)	Ca^{++} Pump Activity (nmol/mg prot/30′)	Distribution of Recovered Calcium Pump Activity (%)
Homogenate	------	0.46 ± .07	------	89.7 ± 6.2	------
Cell Debris, Nuclei, etc.	37.1 ± 1.4	0.40 ± .02	27.3 ± 0.9	22.1 ± 3.0	17.7 ± 0.9
Mitochondria, etc.	11.6 ± 1.6	1.75 ± .46	37.0 ± 3.6	167. ± 28	38.3 ± 2.3
Microsomes, Plasma Membs.	14.4 ± 2.6	1.07 ± .20	28.3 ± 7.3	168. ± 1.5	43.7 ± 3.2
Supernate	36.8 ± 4.0	0.09 ± .03	7.10 ± 2.5	1.50 ± 0.6	1.62 ± .35
Totals	100 %	------	100 %	------	100 %

Table 4. Distribution of Protein, Glucose-6-Phosphatase, and Endoplasmic Reticulum Calcium Pump Activities in Fractions from Freshly Isolated Hepatocytes

Sonicates of freshly isolated hepatocytes were subjected to centrifugation at 1500 x g for ten minutes, 12,000 x g for 20 minutes, and 105,000 x g for one hour to sediment pellets enriched in nuclei, mitochondria, and microsomes, respectively. Protein and enzyme activities were determined in original homogenates and in all fractions, as described in Methods. Data represent means $\pm$ SEM from three cell preparations. Percent recoveries after fractionation were as follows: protein, 71.7 $\pm$ 2.3%; glucose-6-phosphatase, 78.8 $\pm$ 4.4%; endoplasmic reticulum calcium pump, 40.3 $\pm$ 5.4%.

TABLE 4.

DISTRIBUTION OF PROTEIN, GLUCOSE-6-PHOSPHATASE, AND ER CALCIUM PUMP ACTIVITIES IN FRACTIONS FROM FRESHLY ISOLATED HEPATOCYTES

Fraction	Distribution of Recovered Protein (%)	G-6-Pase Activity (umol/mg prot/20′)	Distribution of Recovered G-6-Pase Activity (%)	Ca^{++} Pump Activity (nmol/mg prot/30′)	Distribution of Recovered Calcium Pump Activity (%)
Homogenate	------	0.89 ± .29	------	121. ± 29	------
Cell Debris, Nuclei, etc.	29.0 ± 4.8	0.53 ± .09	16.3 ± 3.8	32.7 ± 2.9	12.3 ± 3.8
Mitochondria, etc.	16.8 ± 3.0	2.00 ± .52	36.0 ± 5.5	120. ± 35	25.7 ± 7.3
Microsomes, Plasma Membs.	25.0 ± 1.2	1.58 ± .50	42.0 ± 2.9	172. ± 24	61.0 ± 4.4
Supernate	29.0 ± 3.1	0.16 ± 0.4	5.00 ± .10	1.41 ± 0.5	1.32 ± 0.6
Totals	100 %	------	100 %	------	100 %

Table 5. Distribution of Protein, Glucose-6-Phosphatase, and Endoplasmic Reticulum Calcium Pump Activities in Fractions from Rat Liver Homogenates

Homogenates of rat liver were subjected to centrifugation at 1500 x g for ten minutes, 12,000 x g for 20 minutes, and 105,000 x g for one hour to sediment pellets enriched in nuclei, mitochondria, and microsomes, respectively. Protein and enzyme activities were determined in original homogenates and in all fractions, as described in Methods. Data represent means $\pm$ SEM from three to four rats. Percent recoveries after fractionation were as follows: protein, 103 $\pm$ 8.1%; glucose-6-phosphatase, 94.5 $\pm$ 4.4%; endoplasmic reticulum calcium pump, 42.0 $\pm$ 1.4%.

TABLE 5.

DISTRIBUTION OF PROTEIN, GLUCOSE-6-PHOSPHATASE, AND ER CALCIUM PUMP ACTIVITIES IN FRACTIONS FROM RAT LIVER HOMOGENATE

Fraction	Distribution of Recovered Protein (%)	G-6-Pase Activity (umol/mg prot/20′)	Distribution of Recovered G-6-Pase Activity (%)	Ca^{++} Pump Activity (nmol/mg prot/30′)	Distribution of Recovered Calcium Pump Activity (%)
Homogenate	------	1.40 ± .27	------	88.0 ± 16	------
Cell Debris, Nuclei, etc.	25.8 ± 1.8	0.65 ± .09	13.5 ± .29	9.44 ± 2.0	7.20 ± 1.1
Mitochondria, etc.	15.4 ± 2.9	1.99 ± .46	22.2 ± 1.4	49.6 ± 15	15.2 ± 4.6
Microsomes, Plasma Membs.	20.7 ± .85	3.28 ± .28	52.5 ± 1.3	122. ± 25	77.7 ± 4.9
Supernate	38.0 ± 3.9	0.44 ± .06	11.5 ± 1.9	1.02 ± .21	1.01 ± .01
Totals	100 %	------	100 %	------	100 %

Ca^{++} pump were observed in microsomes prepared from rat liver homogenates. Enrichments were 1.8- and 1.4-fold in microsomes from fresh hepatocytes, and 2.3- and 1.9-fold in microsomes from cultured hepatocytes for glucose-6-phosphatase and ER Ca^{++} pump activities, respectively. By these criteria, fractions from cultured and fresh hepatocytes closely resembled fractions prepared from rat liver homogenates. In contrast, mitochondrial fractions from cultured cells were enriched in recovered glucose-6-phosphatase (37.0%) and ER Ca^{++} pump (38.3%), relative to rat liver homogenates (22.2% and 15.2%, respectively). Mitochondrial fractions from cultured cells contained high specific activities (i.e. comparable to specific activities found in the microsomal fractions) of these enzymes relative to rat liver homogenate; fresh cells did as well but to a lesser extent. Apparently a significant amount of endoplasmic reticulum sedimented at a lower g force than expected. Perhaps disruption of hepatocytes by sonication produced larger and therefore heavier endoplasmic reticulum fragments than Potter-Elvejeham homogenization produced from liver tissue. Isolated cells do not break when homogenized with a teflon mortar and glass pestle, probably because cells deform and slip around the edges of the pestle. Alternatively, endoplasmic reticulum may have been closely associated with or attached to heavier mitochondria, and therefore sedimented with the mitochondria at 12,000 x g. This hypothesis is consistent with the observations of Chapman et al. (1973). They found rough endoplasmic reticulum in close spatial arrangement with mitochondria in hepatocytes maintained for one day in culture, and speculated that this might be an organelle complex responsible for new membrane formation.

Microsomal Cytochrome P-450 and Cytochrome c Reductase

Microsomal fractions from cultured and fresh hepatocytes had characteristics indicating that they were enriched in endoplasmic reticulum, and so these fractions were further evaluated for cytochrome P-450 content and cytochrome c reductase activity. Cytochrome P-450 content of microsomes obtained from fresh hepatocytes (0.45 nmole/mg protein/minute, Table 6) was lower than but comparable to values previously reported for rat liver microsomes (0.54 nmole/mg protein, Fahl et al., 1979; 0.60 nmole/mg protein, Ueng et al., 1983; 0.73 nmole/mg protein, Alvares and Kappas, 1977). Cytochrome c reductase activity of microsomes obtained from fresh hepatocytes (115 nmole/mg protein/minute, Table 6) was comparable to values previously reported for rat liver microsomes, which varied widely (157 nmole/mg protein/minute, Fahl et al., 1979; 62 nmole/mg protein/minute, Ueng et al., 1983; 118 nmole/mg protein/minute, Alvares and Kappas, 1977). When microsomal fractions from cultured cells were compared to microsomal fractions from freshly isolated cells, 64% of the cytochrome P-450 content remained after 18 hours in culture (Table 6). In hepatocytes maintained for one day in culture, the wavelength of maximum absorbance was not shifted away from 450 nm, suggesting that no qualitative changes in cytochrome P-450 occurred. When cytochrome c reductase activity was determined, 131% of the activity of fresh cells was observed in cultured cells, a statistically non-significant increase. The standard error for cytochrome c reductase activity was somewhat high (individual values were 214, 110, 128 nmole/mg protein/minute). Other investigators have reported no increases in microsomal cytochrome c reductase activity

Table 6. Cytochrome P-450 Content and Cytochrome c Reductase Activity in Microsomal Fractions from Fresh and Cultured Hepatocytes

Microsomal fractions (105,000 x g pellet) were prepared from freshly isolated and cultured hepatocytes. Cytochrome P-450 content and cytochrome c reductase activity were determined as described in Methods. Data represent means $\pm$ SEM from three cell preparations. Differences between fresh and cultured cells are significant at $p \leq 0.05$ (*) by Student's t-test.

TABLE 6.

MICROSOMAL CYTOCHROME P-450 CONTENT AND
CYTOCHROME c REDUCTASE ACTIVITY
IN CULTURED AND FRESH HEPATOCYTES

Preparation	Wavelength$_{max}$ (nm)	Cytochrome P-450 (nmol/mg prot)	Cytochrome c Reductase (nmol/mg prot/min)
Cultured Hepatocytes	450.0 ± 0.4	0.29 ± 0.03	151 ± 32
Fresh Hepatocytes	450.6 ± 0.2	0.45 ± 0.05*	115 ± 2.0

after one day in culture using a variety of media formulations, including conditions similar to those used here (Grant et al., 1985, Fahl et al., 1979, Guzelian et al., 1977).

Comparison of Methods for Halocarbon Exposures

Direct addition to the culture dishes of CCl_4 dissolved in a small amount of ethanol (0.3% addition by volume) was compared to exposure of cultured hepatocytes to CCl_4 vapors generated in a closed chamber in a 37° C room (see Methods). Essentially identical results were obtained for partial and maximal phosphorylase activation and ER Ca^{++} pump inhibition regardless of the exposure method (Figure 11). Direct addition of the compounds was the method of choice for most experiments. By this technique, effective media concentrations were instantly achieved. It was also a simpler method for handling large numbers of samples.

EFFECTS OF CARBON TETRACHLORIDE IN RAT HEPATOCYTE CULTURES

Phosphorylase a and Endoplasmic Reticulum Calcium Pump

Time-dependence of the effects of CCl_4 was investigated in cultured hepatocytes. Activation of phosphorylase and inhibition of the ER Ca^{++} pump were monitored in the presence of 3 mM CCl_4 or vehicle (0.3% ethanol) (Figure 12). Basal phosphorylase a activity averaged 45 nmole/mg protein/minute in control cells throughout the duration of the experiment, which was comparable to the activity found in liver tissue in in vivo studies. Phosphorylase a activity was stimulated to 162 nmole/mg protein/minute after exposure to 3 mM CCl_4 for 2.5 minutes, and to 178 nmole/mg protein/minute after five minutes. Maximum activity was achieved by five minutes. Phosphorylase a activity remained elevated 30 minutes after CCl_4. Differences between phosphorylase a levels in

Figure 11. Exposure of Cultured Hepatocytes to Carbon Tetrachloride as Vapors or by Direct Addition

Carbon tetrachloride (one or two mM) was added directly to the culture dishes and exposures were terminated at five minutes (left panels), or culture dishes were exposed to vapors of carbon tetrachloride (0.3 ml/ liter air) generated in a closed chamber at 37° C for ten or 20 minutes (right panels). Phosphorylase a activity was quantitated as nmoles inorganic phosphate released from glucose-1-phosphate/mg protein/minute. Endoplasmic reticulum calcium pump activity was quantitated as nmoles ^{45}Ca accumulated/mg protein/30 minutes. Data represent means from two hepatocyte preparations.

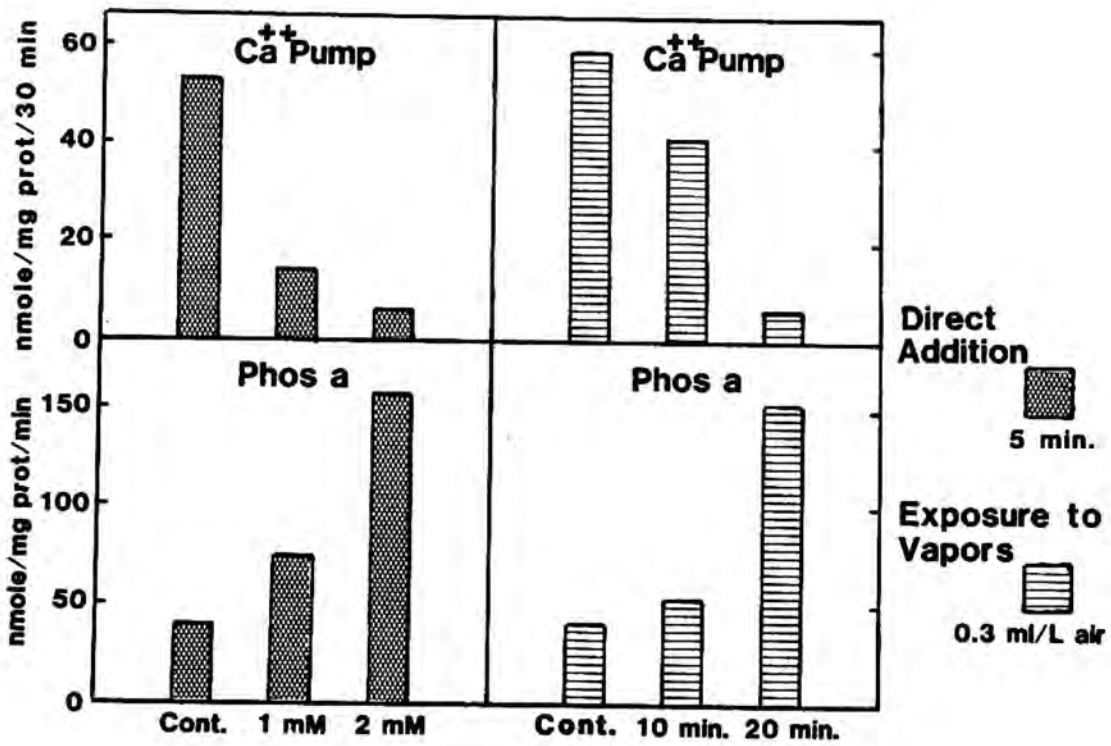
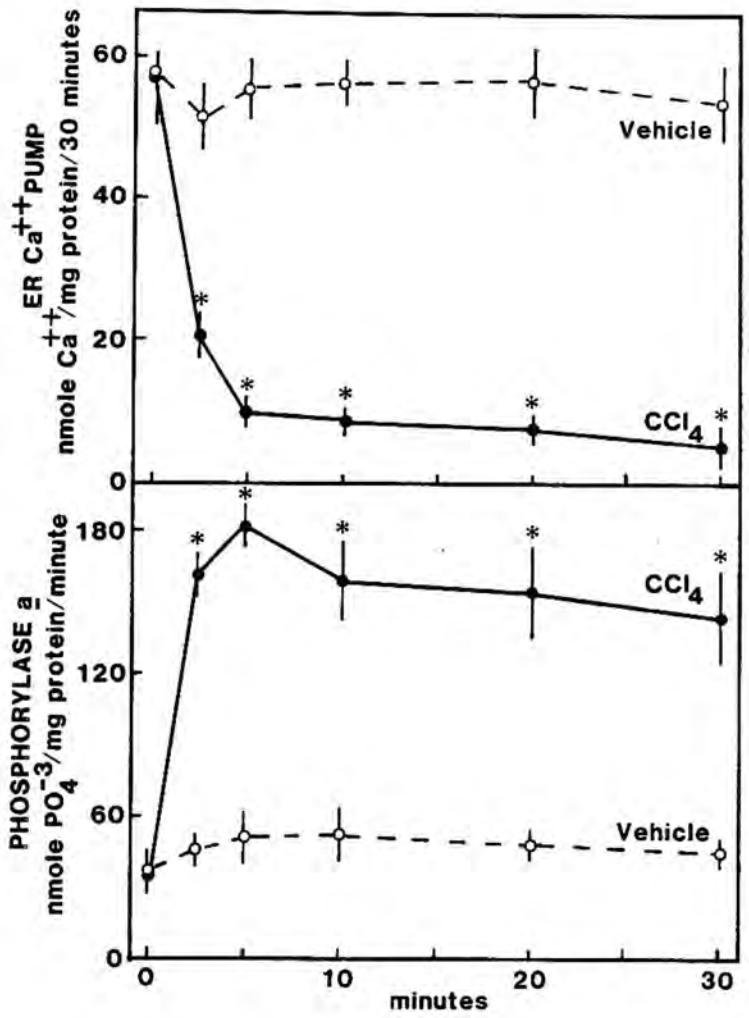

nmole/mg prot/30 min
60
40
20
0
Ca++ Pump
Ca++ Pump
nmole/mg prot/min
150
100
50
0
Phos a
Phos a
Cont.
1 mM
2 mM
Cont.
10 min.
20 min.
Direct Addition
5 min.
Exposure to Vapors
0.3 ml/L air

Figure 12. Time-Dependent Effects of Carbon Tetrachloride on Phosphorylase a and Endoplasmic Reticulum Calcium Pump Activities in Cultured Hepatocytes

Carbon tetrachloride (three mM) or vehicle (ethanol, 0.3% by volume) was added to hepatocyte cultures at zero minutes. Exposures were terminated at 2.5 through 30 minutes. Phosphorylase a activity was quantitated as nmoles inorganic phosphate released from glucose-1-phosphate/mg protein/minute. Endoplasmic reticulum calcium pump activity was quantitated as nmoles ^{45}Ca accumulated/mg protein/30 minutes. Data represent means $\pm$ SEM from three cell preparations. Differences between treatments at each timepoint are significant at $p \leq 0.05$ (*) by Student's t-test.

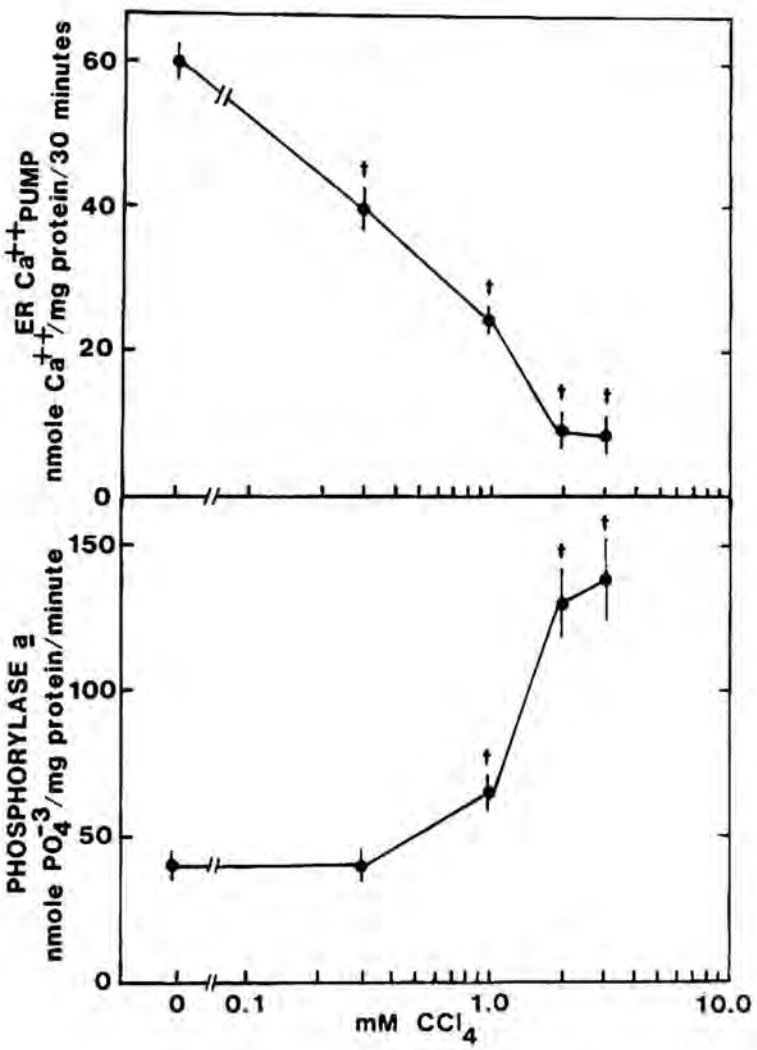

ER Ca++PUMP
nmole Ca++/mg protein/30 minutes
60
40
20
0
Vehicle
CCl4
PHOSPHORYLASE a
nmole PO4-3/mg protein/minute
180
120
60
0
CCl4
Vehicle
0
10
20
30
minutes

control and CCl_4-treated cells were significant at all timepoints. ER Ca^{++} pump activity in hepatocytes averaged 57 nmole/mg protein/30 minutes. This was less than ER Ca^{++} pump activity found in rat liver *in vivo*, indicating that some of this activity was lost from cells either with time in culture, or as a result of sample collection or preparation techniques; further investigation demonstrated that all three likely contributed to the decreased activity. After 2.5 minutes of exposure to 3 mM CCl_4, ER Ca^{++} pump activity was depressed to 19 nmole/mg protein/30 minutes, and after five minutes pump activity was further reduced to 9 nmole/mg protein/30 minutes, nearly maximal inhibition. Differences in ER Ca^{++} pump activities between control and CCl_4-treated cells were significant at all timepoints. On the basis of these results, a five minute exposure period was selected for subsequent experiments, because at this time CCl_4 had essentially achieved its maximal effects.

Dose-dependent effects of CCl_4 were investigated in hepatocyte cultures. Appearance of phosphorylase *a* and ER Ca^{++} pump inhibition at five minutes were determined as a function of CCl_4 concentration (Figure 13). Phosphorylase *a* activity became elevated from 44 to 45, 66, 130, and 139 nmole/mg protein/minute at 0, 0.3, 1, 2, and 3 mM CCl_4, respectively. These differences were significant from 1 to 3 mM CCl_4. ER Ca^{++} pump activity was inhibited from 60 to 40, 26, 9, and 8 nmole/mg protein/30 minutes at 0, 0.3, 1, 2, and 3 mM CCl_4, respectively. These differences were significant at 0.3 mM and greater concentrations of CCl_4. In these cells, total phosphorylase activity (*a*+*b*) was determined to be 142 $\pm$ 6 nmole/mg protein/minute, thus all of the phosphorylase was converted to the *a* form after 3 mM CCl_4. On the basis of these results,

Figure 13. Dose-Dependent Effects of Carbon Tetrachloride on Phosphorylase *a* and Endoplasmic Reticulum Calcium Pump Activities in Cultured Hepatocytes

Carbon tetrachloride (0.3 to three mM) or vehicle (ethanol, 0.3% by volume) was added to hepatocyte cultures. Exposures were terminated at five minutes. Phosphorylase *a* activity was quantitated as nmoles inorganic phosphate released from glucose-1-phosphate/mg protein/minute. Endoplasmic reticulum calcium pump activity was quantitated as nmoles ^{45}Ca accumulated/mg protein/30 minutes. Data represent means $\pm$ SEM from three cell preparations. Differences between carbon tetrachloride and vehicle are significant at $p \leq 0.05$ (+) by least significant difference test following one-way ANOVA.


ER Ca^{++} PUMP
nmole Ca^{++}/mg protein/30 minutes
60
40
20
0
PHOSPHORYLASE a
nmole PO_4^{-3}/mg protein/minute
150
100
50
0
0
0.1
1.0
10.0
mM CCl_4

1 mM and 2 mM CCl_4 were selected for subsequent experiments as doses of CCl_4 that partially and maximally stimulated phosphorylase a levels.

Glucose-6-Phosphatase and 5′-Nucleotidase

Time-dependent alterations in glucose-6-phosphatase and 5′-nucleotidase activities were investigated in hepatocyte cultures exposed to toxic doses of CCl_4. Hepatocyte cultures were exposed for 2.5 to 30 minutes to 2 mM CCl_4 or vehicle (0.3% ethanol). This dose of CCl_4 had previously been determined to cause extensive, irreversible inhibition of the ER Ca^{++} pump and corresponding elevation of phosphorylase a activity. In control cultures, the activity of glucose-6-phosphatase, an enzyme associated with endoplasmic reticulum, averaged 1.67 umole/mg protein/20 minutes, in close agreement with activity observed *in vivo*. Following CCl_4 addition, this enzyme activity was significantly inhibited to 1.18 umole/mg protein/20 minutes at 20 minutes, and maximally inhibited to 1.00 umole/mg protein/20 minutes at 30 minutes (Figure 14). CCl_4-induced inhibition of glucose-6-phosphatase activity did not develop as rapidly or as extensively as inhibition of the ER Ca^{++} pump. Control activity of the plasma membrane enzyme 5′-nucleotidase averaged 1.07 umole/mg protein/20 minutes, which agreed closely with activity observed *in vivo*. This enzyme activity was never significantly inhibited in hepatocytes exposed to CCl_4 (Figure 15), as had been found *in vivo*. Table 7 summarizes enzymatic determinations made in liver from rats given 1.5 ml/kg CCl_4 and cultured hepatocytes exposed to 2 to 3 mM CCl_4. Following an initial 15 minute absorptive phase in rats, biochemical alterations caused by CCl_4 developed in the same succcession *in vivo* and *in vitro*, although changes developed much more rapidly in hepatocytes.

Figure 14. Time-Dependent Effects of Carbon Tetrachloride on Glucose-6-Phosphatase Activity in Cultured Hepatocytes

Carbon tetrachloride (two mM) or vehicle (ethanol, 0.3% by volume) was added to hepatocyte cultures at zero minutes. Exposures were terminated at 2.5 through 30 minutes. Glucose-6-phosphatase activity was quantitated as umoles inorganic phosphate released from glucose-6-phosphate/mg protein/20 minutes. Data represent means $\pm$ SEM from three cell preparations. Differences between treatments at each timepoint are significant at $p \leq 0.05$ (*) by Student's t-test.

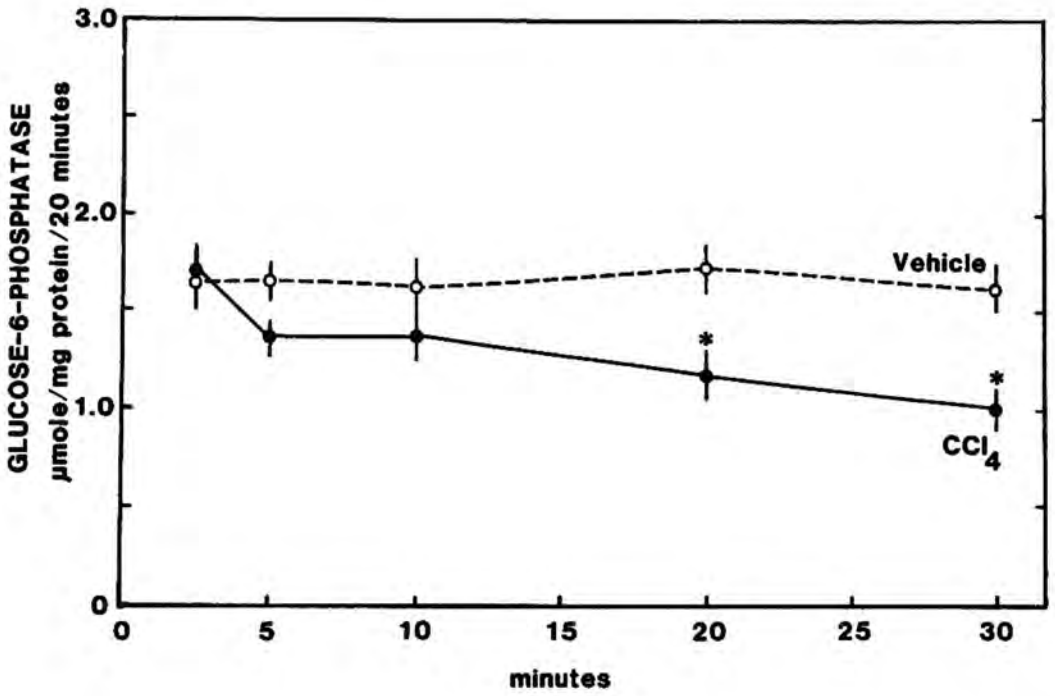

GLUCOSE-6-PHOSPHATASE
µmole/mg protein/20 minutes
3.0
2.0
1.0
0
0
5
10
15
20
25
30
minutes
Vehicle
CCl4
*
*

Figure 15. Time-Dependent Effects of Carbon Tetrachloride on 5′-Nucleotidase Activity in Cultured Hepatocytes

Carbon tetrachloride (two mM) or vehicle (ethanol, 0.3% by volume) was added to hepatocyte cultures at zero minutes. Exposures were terminated at 2.5 through 30 minutes. 5′-Nucleotidase activity was quantitated as umoles inorganic phosphate released from adenosine monophosphate/mg protein/20 minutes. Data represent means ± SEM from three cell preparations. Differences between treatments at each timepoint are not significant by Student′s t-test.

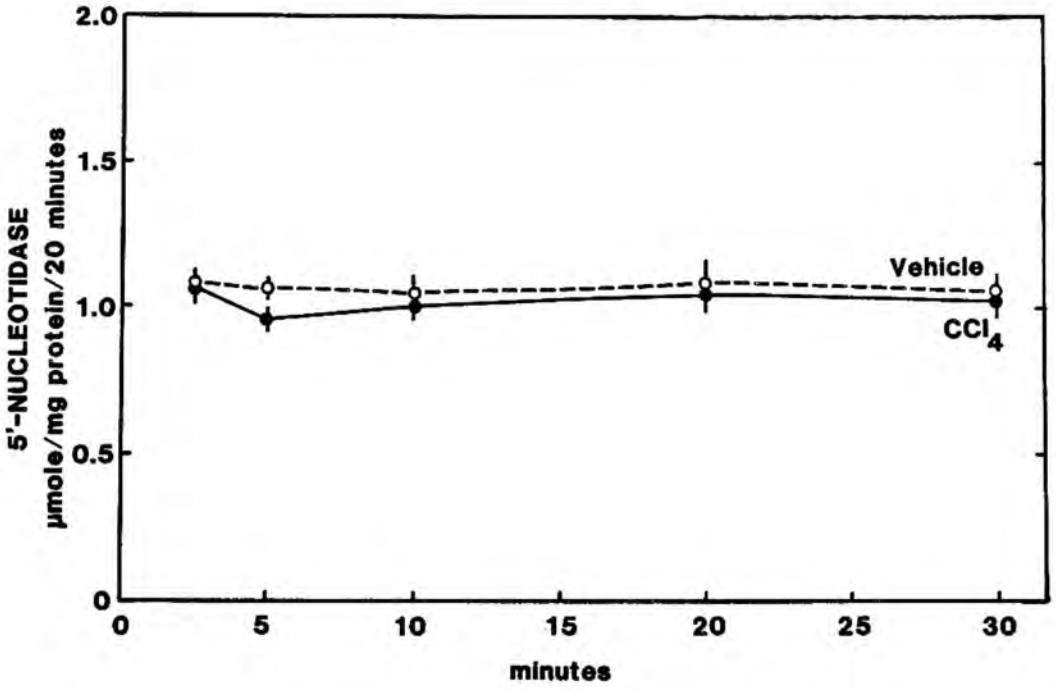

2.0
1.5
1.0
0.5
0
5'-NUCLEOTIDASE
µmole/mg protein/20 minutes
0
5
10
15
20
25
30
minutes
Vehicle
CCl4

Table 7. Carbon Tetrachloride-Induced Alterations in Enzyme Activities *In Vivo* and in Cultured Hepatocytes

Rats received carbon tetrachloride (1.5 ml/kg) or vehicle (corn oil) at zero minutes for *in vivo* studies. Carbon tetrachloride or vehicle (ethanol) was added directly to hepatocyte cultures (2 mM for glucose-6-phosphatase and 5′-nucleotidase, 3 mM for all other determinations) at zero minutes for *in vitro* studies. Liver or hepatocyte samples were processed at the indicated times, and enzyme activities were determined as described in Methods. Results are expressed as percent of activity obtained in controls for each timepoint. Data represent means ± SEM from three to five animals *in vivo*, and means ± SEM from three to four cell preparations *in vitro*. Control enzyme activities were as follows:

Rat Liver:

Phosphorylase a	31.1 ± 1.9 nmol/mg prot/min
ER Calcium Pump	191 ± 11 nmol/mg prot/30 min
Glucose-6-Phosphatase	1.59 ± 0.04 umol/mg prot/20 min
5′-Nucleotidase	0.99 ± 0.03 umol/mg prot/20 min
Glutamic-Pyruvic Transaminase	19.6 ± 1.4 IU/liter

Rat Hepatocyte Cultures:

Phosphorylase a	33.1 ± 1.6 nmol/mg prot/min
ER Calcium Pump	61.8 ± 3.2 nmol/mg prot/30 min
Glucose-6-Phosphatase	1.67 ± 0.09 umol/mg prot/20 min
5′-Nucleotidase	1.07 ± 0.03 umol/mg prot/20 min
Glutamic-Pyruvic Transaminase	3.61 ± 0.71 % released

TABLE 7.

CARBON TETRACHLORIDE-INDUCED ENZYME ALTERATIONS
(control activity=100%)

RAT LIVER
(% of control)

	15 min. %	30 min. %	60 min. %	120 min. %	8 hrs. %
Phosphorylase a	93 ± 3	136 ± 19	139 ± 5	137 ± 19	195 ± 14
ER Calcium Pump	96 ± 13	35 ± 4	21 ± 2	17 ± 3	6 ± 1
Glucose-6-Phos.	103 ± 5	80 ± 7	81 ± 5	71 ± 4	43 ± 5
5′-Nucleotidase	98 ± 3	92 ± 10	101 ± 9	98 ± 6	96 ± 7
Glut.-Pyr. Trans.	106 ± 24	104 ± 14	100 ± 24	132 ± 5	939 ± 364

RAT HEPATOCYTE CULTURES
(% of control)

	2.5 min. %	5 min. %	10 min. %	20 min. %	30 min. %
Phosphorylase a	472 ± 18	392 ± 25	272 ± 22	272 ± 30	280 ± 15
ER Calcium Pump	41 ± 7	19 ± 3	13 ± 4	13 ± 3	10 ± 2
Glucose-6-Phos.	104 ± 3	83 ± 2	83 ± 2	71 ± 4	60 ± 4
5′-Nucleotidase	98 ± 6	86 ± 5	95 ± 4	102 ± 6	97 ± 3
Glut.-Pyr. Trans.	112 ± 15	218 ± 62	792 ± 60 (@ 15 min.)		909 ± 126

Glutamic-Pyruvic Transaminase Release

Release of the cytoplasmic enzyme glutamic-pyruvic transaminase was investigated in cultured hepatocytes (Figure 16). In cells exposed to vehicle (0.3% ethanol) or 1 mM CCl_4, four to seven percent of the total quantity of this intracellular enzyme appeared in the culture medium over four hours. By as early as 15 minutes, statistically significant release of total cellular glutamic-pyruvic transaminase occurred after 2 mM CCl_4 (10%), and even greater release occurred after 3 mM CCl_4 (26%) and 4 mM CCl_4 (52%). Medium levels of released enzyme were maximal by 30 minutes for all doses of CCl_4 examined and remained elevated through four hours after CCl_4 addition. Thus, evidence of hepatotoxic damage was obtained by 30 minutes at doses of CCl_4 that caused phosphorylase activation (2 mM and 3 mM). An even higher dose of CCl_4 (4 mM) was required to release massive amounts of this cytosolic enzyme (66%) than was required for maximal phosphorylase activation. Subsequent experiments have confirmed that progressive time-dependent effects of CCl_4 on glutamic-pyruvic transaminase release are more difficult to demonstrate in hepatocyte cultures than dose-dependent effects (R. M. Long, unpublished).

Adenosine 3′:5′-Cyclic Monophosphate

Cyclic AMP content at 2.5 minutes and phosphorylase *a* activity at five minutes were determined in hepatocytes exposed to CCl_4 or other agents known to increase phosphorylase *a* activity by either cAMP or Ca^{++}. Phosphorylase *a* was maximally elevated from 35 to 132 nmole/mg protein/minute after exposure of cells to the peptide hormone glucagon or the beta-adrenergic agent isoproterenol (Figure 17). These changes

Figure 16. Glutamic-Pyruvic Transaminase Activity in Medium from Hepatocyte Cultures Exposed to Carbon Tetrachloride

Carbon tetrachloride (one to four mM) or vehicle (ethanol, 0.3% by volume) was added to hepatocyte cultures at zero minutes. Glutamic-pyruvic transaminase activity was quantitated in medium sampled at 15 through 240 minutes by monitoring oxidation of NADPH spectrophotometrically. Results were expressed as per cent of total cellular enzyme released into the culture medium. This was calculated as [(100)x(units in medium)]/[(units in medium)+(units remaining in hepatocyte homogenate)]. Data represent means $\pm$ SEM from three cell preparations. Differences between treatments and vehicle at each timepoint are significant at $p \leq 0.05$ (+) by least significant difference test following two-way ANOVA.

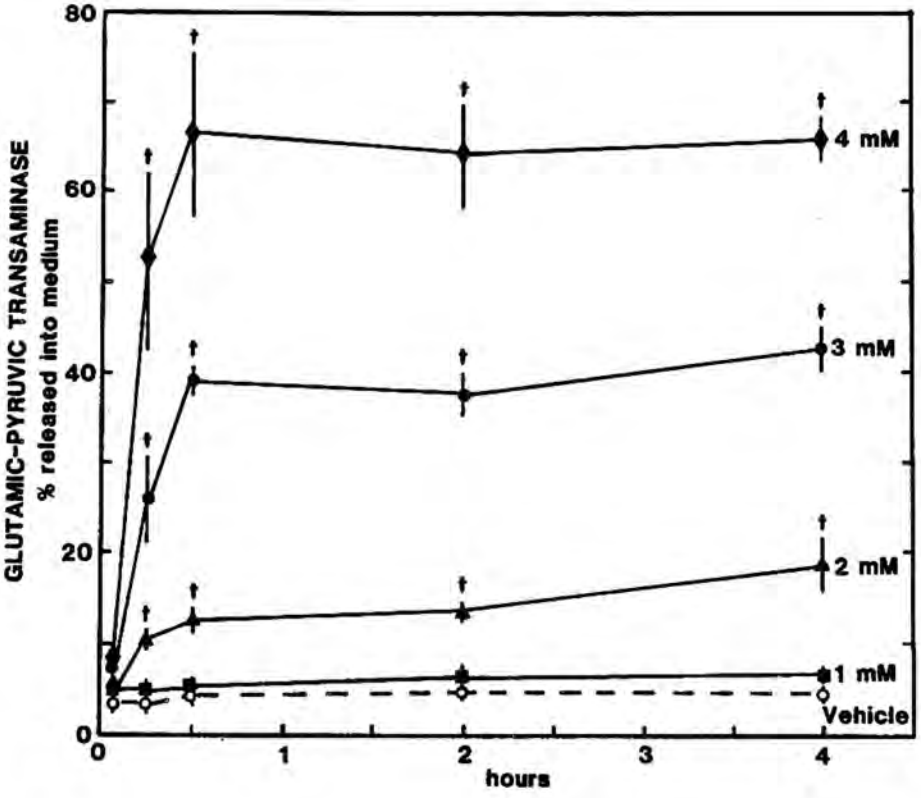
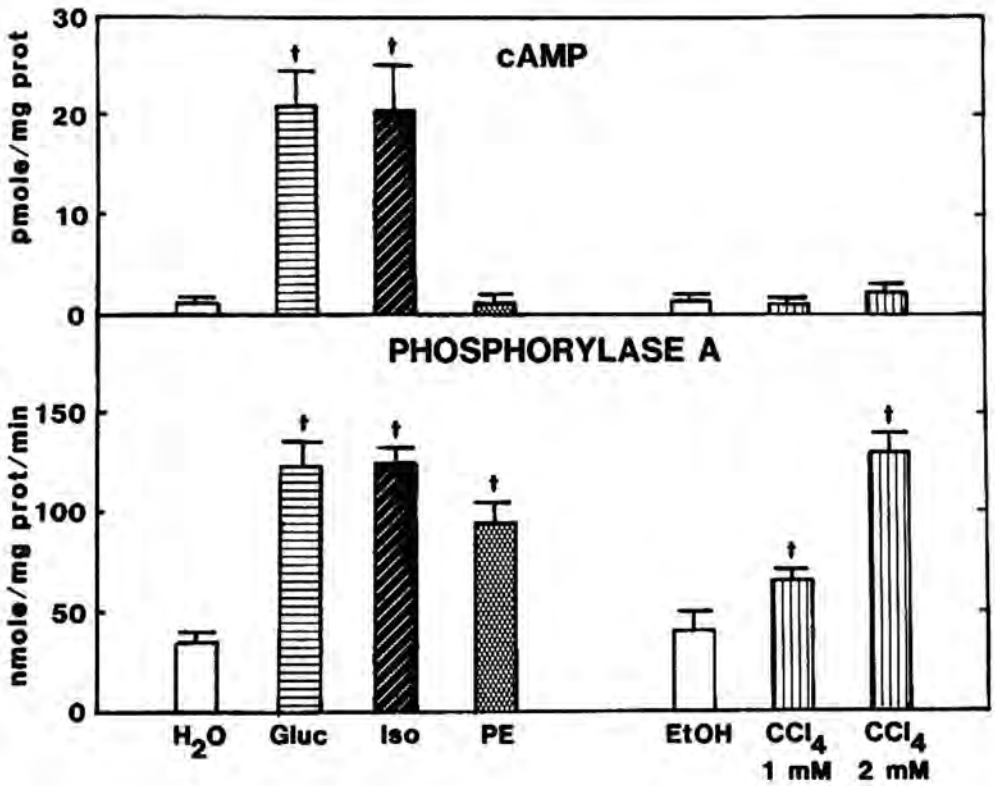

GLUTAMIC-PYRUVIC TRANSAMINASE
% released into medium
80
60
40
20
0
0
1
2
3
4
hours
4 mM
3 mM
2 mM
1 mM
Vehicle

Figure 17. Cyclic AMP Content and Phosphorylase a Activity in Cultured Hepatocytes Exposed to Glucagon, Isoproterenol, Phenylephrine and Carbon Tetrachloride

Hepatocyte cAMP content (pmole cAMP/mg protein) was determined at 2.5 minutes, and phosphorylase a activity (nmoles phosphate released from glucose-1-phosphate/mg protein/minute) was determined at five minutes after addition of glucagon (Gluc, 10 nM), isoproterenol (Iso, 10 uM), phenylephrine (PE, 10 uM), carbon tetrachloride (one or two mM), and their respective vehicles (water or ethanol). Data represent means $\pm$ SEM from three cell preparations. Differences between treatments and their controls are significant at $p \leq 0.05$ (+) by least significant difference tests following one-way ANOVAs.


cAMP
pmole/mg prot
30
20
10
0
PHOSPHORYLASE A
nmole/mg prot/min
150
100
50
0
H2O
Gluc
Iso
PE
EtOH
CCl4 1 mM
CCl4 2 mM

were accompanied by 20-fold increases in cellular cAMP content. Control levels of cAMP in unstimulated cells averaged 1.2 pmole/mg protein, which was comparable to levels found *in vivo*. Phenylephrine, an alpha-adrenergic agent that mobilizes liver cell Ca^{++} via increased inositol trisphosphates, increased phosphorylase *a* activity to 98 nmole/mg protein/minute without increasing cAMP concentrations. These agents served as positive controls to indicate that cultured hepatocytes were functioning as expected, and that the changes could be measured. Doses of CCl_4 that caused partial (1 mM) and maximal (2 mM) phosphorylase activation were investigated. One mM CCl_4 increased phosphorylase *a* activity to 66 nmole/mg protein/minute, and 2 mM CCl_4 increased phosphorylase *a* activity to 129 nmole/mg protein/minute. Neither dose of CCl_4 significantly increased hepatocyte cAMP content. Thus, as had been found *in vivo*, CCl_4-induced phosphorylase activation occurred in the absence of elevated cAMP concentrations. These results were consistent with the interpretation that appearance of phosphorylase *a* was Ca^{++}-mediated. Undetectably small increases in cAMP may have been able to cause phosphorylase conversion to the *a* form. However, isoproterenol cannot cause maximal stimulation of liver phosphorylase in the absence of large changes in cAMP content (Morgan *et al.*, 1983). The possibility that a very small increase in cAMP can synergize with the effect of Ca^{++} has not been excluded.

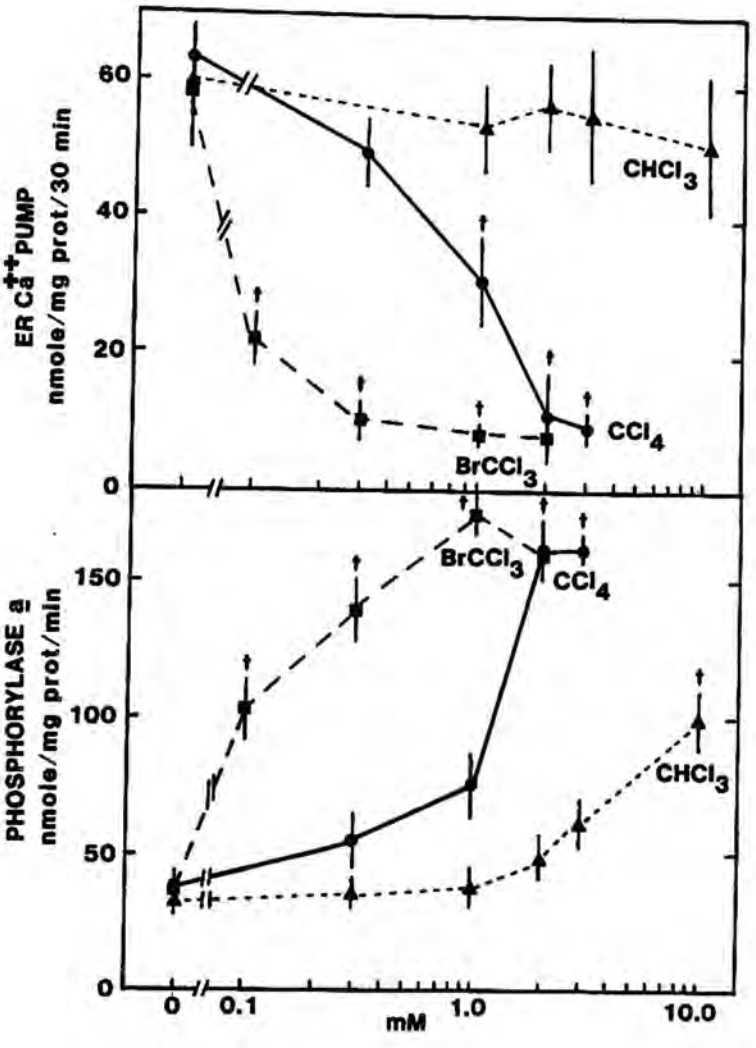
Halogenated Analogs of Carbon Tetrachloride

Bromotrichloromethane ($BrCCl_3$) and chloroform ($CHCl_3$), halogenated congeners of CCl_4, were characterized in dose/response experiments with cultured hepatocytes. $BrCCl_3$ is eight to ten times as potent as CCl_4 *in vivo* (Waller *et al.*, 1983), and $CHCl_3$ is approximately one-tenth as

potent as CCl_4 *in vivo* (Klaassen and Plaa, 1969, Slater and Sawyer, 1971). These halocarbons all have comparable physical properties, including polarity index, boiling point, and oil/water partition coefficient (Sato and Nakajima, 1979, Krstulovic and Brown, 1982). Effects of these haloalkanes on phosphorylase activation and ER Ca^{++} pump inhibition were examined in hepatocyte cultures (Figure 18). Shifts of the dose/response curves indicated that the relative potencies of these analogs of CCl_4 approximated that which had been found *in vivo*. One-tenth mM $BrCCl_3$ was almost equipotent with 1.0 mM CCl_4 for phosphorylase activation (2.8- and 2.2-fold controls, respectively) and ER Ca^{++} pump inhibition (0.4- and 0.5-fold controls, respectively). Ten mM $CHCl_3$ did not significantly inhibit the ER Ca^{++} pump (0.9-fold control), but paradoxically did cause phosphorylase activation (2.8-fold control). This could have occurred because 10 mM $CHCl_3$ did not stay in solution; droplets of $CHCl_3$ collected at the bottom of the culture dishes. $CHCl_3$ may have directly perturbed the plasma membrane, allowing extracellular Ca^{++} to enter and increase cytoplasmic Ca^{++} concentrations before inhibition of the ER Ca^{++} pump developed. Alternatively, $CHCl_3$ may have directly affected the phosphorylase enzyme by allosterically activating the *b* form, or by causing its conversion to the *a* form independent of Ca^{++}. The 10-fold shifted dose/response curves following $BrCCl_3$ provided the best evidence that phosphorylase activation and ER Ca^{++} pump inhibition were not non-specific solvent effects, and in fact resulted from the hepatotoxic properties of these haloalkanes. The same rank order of potency for release of intracellular enzymes from hepatocyte suspensions by $BrCCl_3$, CCl_4, and $CHCl_3$ was demonstrated by Tyson *et al*. (1983).

Figure 18. Dose-Dependent Effects of Bromotrichloromethane, Carbon Tetrachloride, and Chloroform on Phosphorylase *a* and Endoplasmic Reticulum Calcium Pump Activities in Cultured Hepatocytes

Bromotrichloromethane, carbon tetrachloride, chloroform, or vehicle (ethanol, 0.3% by volume) was added to hepatocyte cultures. Exposures were terminated at five minutes. Phosphorylase *a* activity was quantitated as nmoles inorganic phosphate released from glucose-1-phosphate/mg protein/minute. Endoplasmic reticulum calcium pump activity was quantitated as nmoles ^{45}Ca accumulated/mg protein/30 minutes. Data represent means $\pm$ SEM from three cell preparations. Differences between treatments and vehicle are significant at $p \leq 0.05$ (+) by least significant difference tests following one-way ANOVAs.


ER Ca++ PUMP
nmole/mg prot/30 min
60
40
20
0
CHCl3
CCl4
BrCCl3
PHOSPHORYLASE a
nmole/mg prot/min
150
100
50
0
BrCCl3
CCl4
CHCl3
0
0.1
mM
1.0
10.0

Protection of Calcium Pump and Phosphorylase a

In order to investigate the dependence of phosphorylase activation and ER Ca^{++} pump inhibition upon metabolic processes (including cytochrome P-450-dependent metabolism), hepatocyte cultures were maintained on ice before and during exposure to CCl_4 (Table 8). Basal phosphorylase activity in cells kept at 0-4° C was depressed slightly, relative to that found in hepatocytes maintained at 37° C (39 vs. 46 nmole/mg protein/minute). After 1 mM or 2 mM CCl_4, increased phosphorylase *a* activity did not occur in cells kept on ice (38 and 36 nmole/mg protein/minute, respectively). ER Ca^{++} pump activity in hepatocytes kept on ice was depressed slightly (43 vs. 53 nmole/mg protein/30 minutes). The ER Ca^{++} pump was almost completely protected at 0-4° C from inhibition by 1 mM or 2 mM CCl_4 (41 and 37 nmole/mg protein/30 minutes, respectively). The protective effect of incubation on ice was significant for both phosphorylase activation and pump inhibition, when compared to results obtained at 37° C. This suggested either that metabolic activation of CCl_4 by cytochrome P-450 was prevented, or that expression of ER Ca^{++} pump inhibition and phosphorylase activation that follows metabolic activation was blocked.

Metabolism Inhibitors and Inducers

Attempts were made to manipulate CCl_4-induced changes in phosphorylase *a* activity through the use of various inhibitors of drug metabolizing enzymes, including SKF-525A, chloramphenicol, cimetidine, piperonyl butoxide, and carbon monoxide. Preliminary results indicated that effective concentrations of these agents could not be achieved in cells at doses that did not cause what were interpreted as non-specific

Table 8. Phosphorylase a and Endoplasmic Reticulum Calcium Pump Activities in Cultured Hepatocytes Maintained at 37° C and On Ice During Exposure to Carbon Tetrachloride

Cultured hepatocytes were maintained at 37° C or on ice (0-4° C) for five minutes prior to halocarbon addition. Carbon tetrachloride (1 or 2 mM) or vehicle (ethanol, 0.3% by volume) was added to hepatocyte cultures, and exposures were terminated at five minutes. Phosphorylase a activity was quantitated as nmoles inorganic phosphate released from glucose-1-phosphate/mg protein/minute, and ER calcium pump activity was determined as nmoles ^{45}Ca accumulated/mg protein/30 minutes. Data represent means ± SEM from three cell preparations. Differences between carbon tetrachloride and vehicle are significant (+) and between temperatures are significant (++) at $p \leq 0.05$ by least significant difference tests following two-way ANOVA.

TABLE 8.

PHOSPHORYLASE a AND ENDOPLASMIC RETICULUM CALCIUM PUMP ACTIVITIES IN CULTURED HEPATOCYTES MAINTAINED AT 37° C AND ON ICE

	Phosphorylase a (nmol/mg prot/min)		ER Calcium Pump (nmol/mg prot/30 min)	
	37° C	0 - 4° C	37° C	0 - 4° C
Vehicle (ethanol)	46 ± 5	39 ± 7	53 ± 9	43 ± 4
CCl_4 (1 mM)	66 ± 8 $^{+}$	38 ± 7^{++}	14 ± 1^{+}	41 ± 4^{++}
CCl_4 (2 mM)	133 ± 18^{+}	36 ± 7^{++}	7 ± 2^{+}	37 ± 8^{++}

changes in phosphorylase a activity (R. M. Long, unpublished). Joseph et al. (1985) have reported that structurally similar compounds can activate phosphorylase in a non-Ca^{++}-mediated manner.

Dose/response experiments were performed evaluating ER Ca^{++} pump inhibition and phosphorylase activation in cultured hepatocytes isolated from rats that had been pretreated with agents that enhance CCl_4 hepatotoxicity. Hepatocytes were prepared from rats given phenobarbital (75 mg/kg/day for 5 days), which potentiates the release of intracellular enzymes (Garner and McLean, 1969) and ER Ca^{++} pump inhibition (L. Moore, unpublished) produced by CCl_4 in vivo. Cytochrome P-450 levels were three- to four-fold elevated in cells isolated from phenobarbital-treated rats (R. M. Long, unpublished). Cultured hepatocytes were also prepared from rats that received 2,5-hexanedione. This compound was reported to potentiate CCl_4 hepatotoxicity as monitored by intracellular enzyme release both in rat liver in vivo (Jernigan and Harbison, 1982) and in hepatocytes isolated from pretreated rats (Jernigan et al., 1983). Neither phenobarbital nor hexanedione pretreatment shifted the response curves for ER Ca^{++} pump inhibition or phosphorylase activation to lower doses of CCl_4 (R. M. Long, unpublished). Thus, it could not be demonstrated that phosphorylase activation and ER Ca^{++} pump inhibition in cultured hepatocytes depended upon metabolic activation of CCl_4, as would be predicted from studies of CCl_4 hepatotoxicity in vivo.

Calibration Curve for Phosphorylase a Activity vs. Calcium

To relate increases in phosphorylase a activity to internal Ca^{++} concentrations in cultured rat hepatocytes, a calibration curve was constructed using a method described for freshly isolated guinea pig hepatocyte suspensions by Burgess et al. (1983). Hepatocytes were made

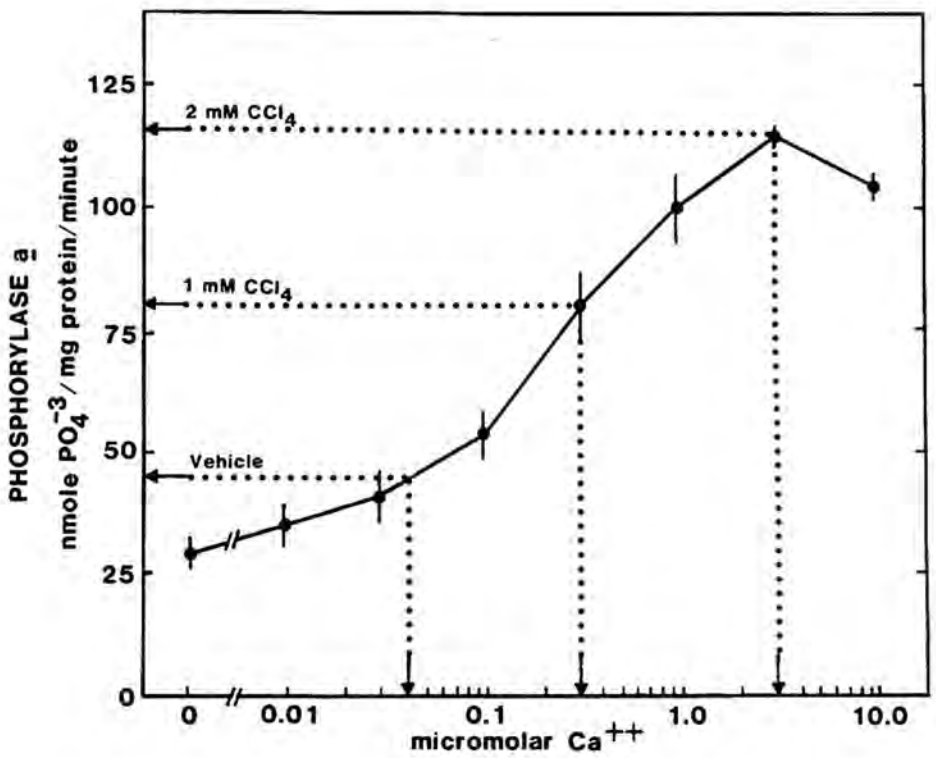
permeable to small molecules and ions in a saponin-containing, cytosol-like medium. Cells were then exposed to a range of Ca^{++} concentrations appropriately buffered with EGTA (see Methods). Phosphorylase *a* activity measured in these cells increased as an approximately linear function of the logarithm of the Ca^{++} concentration over the range of 0.01 uM to $\geq$3 uM Ca^{++} (Figure 19). In intact (non-permeabilized) cultured hepatocytes, the resting Ca^{++} concentration was estimated from the basal phosphorylase *a* activity and the calibration curve to be 0.04 uM Ca^{++}. In hepatocytes exposed to 1 mM and 2 mM CCl_4, the Ca^{++} concentrations were estimated to be 0.3 uM and $\geq$3 uM Ca^{++}, respectively, as interpolated from the phosphorylase *a* activities determined in these cells. Thus, Ca^{++} concentrations were estimated to be at least 100-fold elevated over unstimulated levels by 2 mM CCl_4. It was noted that phosphorylase *a* activities achieved in hepatocytes exposed to CCl_4 (ranging from 150 to 180 nmole/mg protein/minute, Figures 12 and 13) exceeded maximum phosphorylase *a* activity obtained in permeabilized hepatocytes exposed to Ca^{++}/EGTA buffers (115 nmole/mg protein/minute, Figure 19). Possibly, optimal Ca^{++} concentrations for phosphorylase activation occur over a very narrow range (*e.g.* between 3 and 10 uM) of Ca^{++} concentrations not specifically determined in the calibration curve. Alternatively, perhaps Ca^{++} alone does not fully activate phosphorylase, and other cellular changes are required for complete conversion to the *a* form.

Phosphorylase a in Calcium-Free Medium

To determine whether extracellular sources of Ca^{++} contributed to elevated phosphorylase *a* levels observed in CCl_4-treated hepatocytes,

Figure 19. Calibration Curve for Phosphorylase a Activity vs. Calcium Concentration in Saponin-Treated Cultured Hepatocytes

Cultured hepatocytes were made permeable to small molecules and ions by exposure to a saponin-containing (50 ug/ml), cytosol-like medium for ten minutes. Cells were subsequently exposed for ten minutes to a range of Ca^{++} concentrations appropriately buffered with EGTA. Phosphorylase a activity was then determined as described in Methods. Data represent means $\pm$ SEM from three cell preparations. Dotted lines indicate calcium concentrations interpolated from phosphorylase a activities obtained from cultured hepatocytes exposed to vehicle (ethanol), 1 mM and 2 mM carbon tetrachloride.


PHOSPHORYLASE a
nmole PO_4^{-3}/mg protein/minute
125
100
75
50
25
0
2 mM CCl_4
1 mM CCl_4
Vehicle
0
0.01
0.1
1.0
10.0
micromolar Ca^{++}

cells were exposed to CCl_4 in EGTA-supplemented medium without Ca^{++}. This modified medium was estimated to contain $\leq$1 nM Ca^{++}, and thus was essentially Ca^{++}-free. This estimate was based upon the assumption that total Ca^{++} in deionized water ranges from 0.5 to 1 uM; calculation was performed as described in Methods using the Ca^{++}/EGTA dissociation constant of Martell and Smith (1974). Data in Table 9 illustrate that a CCl_4-induced increase in phosphorylase a activity in Ca^{++}-free medium (125 nmole/mg protein/minute) was slightly but not significantly diminished from that observed in Ca^{++}-containing medium (152 nmole/mg protein/minute). Thus, the presence of extracellular Ca^{++} was not required for phosphorylase activation. Mobilization of internal Ca^{++} stores was sufficient to elevate phosphorylase a activity in hepatocytes exposed to CCl_4. This is consistent with the observation that CCl_4 administered in vivo releases a pool of microsomal Ca^{++} (Reynolds et al., 1972, Moore, 1981).

Quin2 Determination of Intracellular Calcium in Hepatocytes

To corroborate increases in Ca^{++} in liver cells exposed to CCl_4 as suggested by phosphorylase a data, studies were undertaken with an independent methodology. The fluorescent Ca^{++} indicator compound quin2 (Tsien et al., 1982) was used to investigate CCl_4's effects in hepatocyte suspensions. Freshly isolated hepatocyte suspensions were used because they provided a convenient system for use in a fluorometer cuvette, and because quin2-loaded cells had sufficient fluorescent signal and sensitivity (unobtainable with a monolayer of cultured hepatocytes) for these experiments. In hepatocyte suspensions, resting Ca^{++} concentrations averaged 247 $\pm$ 26 nM for all cell preparations examined.

Table 9. Phosphorylase *a* Activity in Cultured Hepatocytes Maintained in Calcium-Containing or Calcium-Free Medium During Exposure to Carbon Tetrachloride

Cultured hepatocytes were maintained in calcium-containing medium (1.8 mM calcium) or calcium-free medium (50 uM EGTA, no added calcium) for fifteen minutes prior to halocarbon addition. Carbon tetrachloride (1 or 2 mM) or vehicle (ethanol, 0.3% by volume) was added to hepatocyte cultures, and exposures were terminated at five minutes. Phosphorylase *a* activity was quantitated as nmoles inorganic phosphate released from glucose-1-phosphate/mg protein/minute. Data represent means $\pm$ SEM from three cell preparations. Differences between carbon tetrachloride and vehicle are significant (+) and between calcium-containing and calcium-free media are not significant at $p \leq 0.05$ by least significant difference tests following two-way ANOVA.

TABLE 9.

PHOSPHORYLASE a ACTIVITY IN CULTURED HEPATOCYTES
IN CALCIUM-CONTAINING AND CALCIUM-FREE MEDIUM

(nmol/mg prot/min)

	Calcium-Containing Medium	Calcium-Free Medium
Vehicle (ethanol)	34 ± 6	54 ± 4
CCl_4 (1 mM)	81 ± 26	67 ± 10
CCl_4 (2 mM)	152 ± 21$^+$	125 ± 19$^+$

This was in good agreement with resting Ca^{++} concentrations obtained by others by a variey of techniques (Charest et al., 1983, estimated 200 nM with quin2; Murphy et al, 1980, estimated 190 nM with arsenazoIII; Burgess et al., 1983, estimated 180 nM from phosphorylase a activity). Interestingly, the resting Ca^{++} concentration found in freshly isolated hepatocyte suspensions (246 nM) was higher than that estimated in primary cultures of hepatocytes from phosphorylase a activity (40 nM). This difference could be explained as restoration of the hepatocyte plasma membrane to greater impermeability to extracellular Ca^{++} with time in culture.

A complete record of an experiment with quin2-loaded cells and unloaded cells exposed to phenylephrine (alpha-adrenergic agent), including calibration procedures of Tsien et al. (1982), is shown in Figure 20. While this was a somewhat extreme example, it illustrates that corrections had to be made for changes in cell autofluorescence caused by the detergent digitonin. In all experiments, arbitrary fluorescence units were corrected for changes in fluorescence that occurred in parallel determinations with unloaded cells, and Ca^{++} concentrations were calculated as described in Methods. Records of experiments with 2 mM CCl_4 and 10 uM phenylephrine, including calibration procedures, are displayed in Figure 21. In the five cell preparations examined, cytosolic Ca^{++} rose to 829 $\pm$ 129 nM after 2 mM CCl_4. In other samples from the same cell preparations, Ca^{++} rose to 997 $\pm$ 136 nM after 10 uM phenylephrine. These changes occurred rapidly and were maximal within twenty seconds. Elevations in Ca^{++} were significant by paired Student's t-test; each cell preparation was evaluated before and after treatment with CCl_4 or phenylephrine. The vehicle (0.3% ethanol) did not affect

Figure 20. Quin2-loaded Cells and Unloaded Cells Exposed to Phenylephrine

Hepatocytes were incubated in Krebs-Henseleit buffer containing 50 uM quin2-AM or vehicle (dimethyl sulfoxide) for five minutes at 37° C. Cells were then recovered by centrifugation and resuspended in fresh medium without quin2. Fluorescence was monitored at excitation wavelength 339 nm and emission wavelength 500 nm; slit widths were 5 nm. Phenylephrine (10 uM), digitonin (20 uM), and EGTA (4 mM) were added successively. Vertical lines indicate opening and closing of the sample compartment door for additions. Parallel manipulations were performed in all experiments with quin2-loaded and unloaded cells in order to correct net fluorescence for changes in cell autofluorescence caused by digitonin. Responses varied between cell preparations; this is an extreme example.

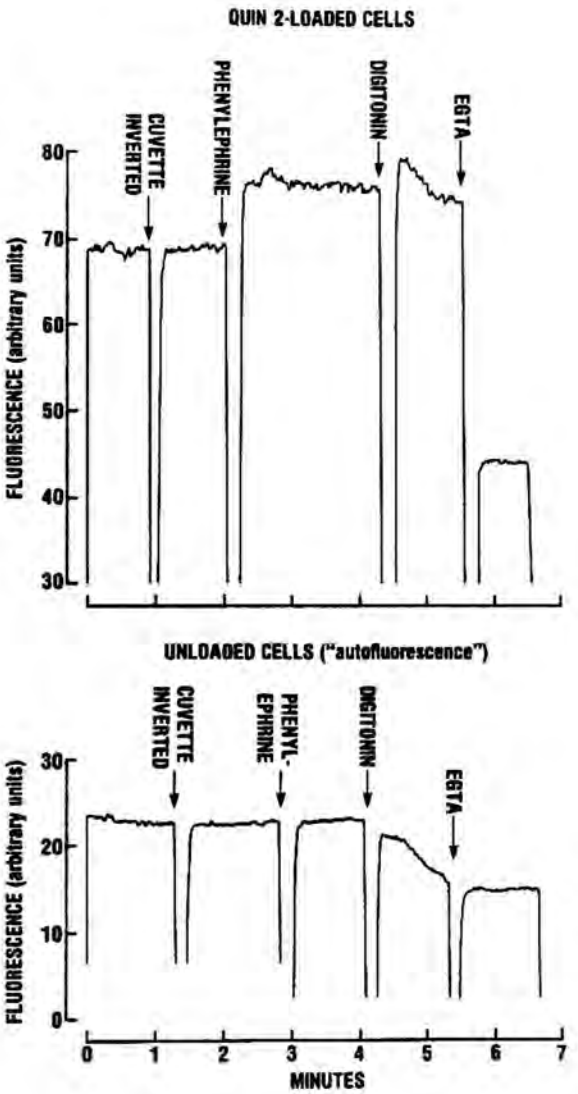
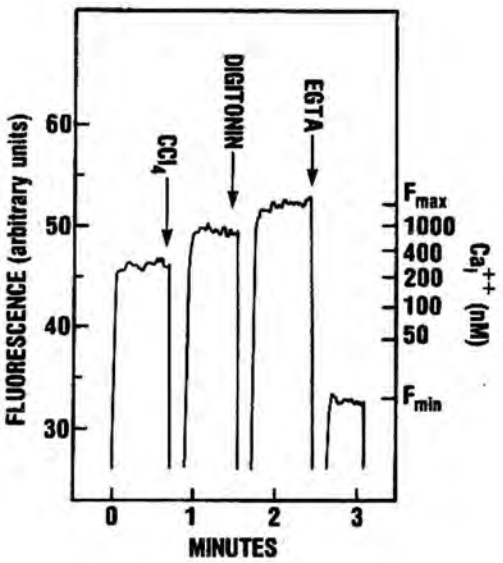
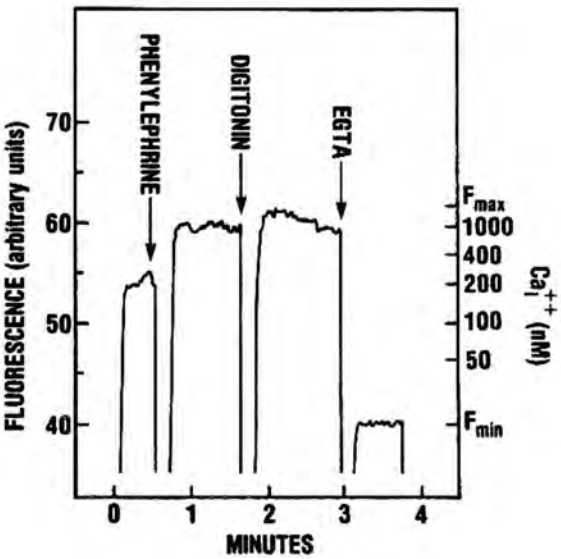

QUIN 2-LOADED CELLS
CUVETTE INVERTED
PHENYLEPHRINE
DIGITONIN
EGTA
FLUORESCENCE (arbitrary units)
80
70
60
50
40
30
UNLOADED CELLS ("autofluorescence")
CUVETTE INVERTED
PHENYL-EPHRINE
DIGITONIN
EGTA
FLUORESCENCE (arbitrary units)
30
20
10
0
0
1
2
3
4
5
6
7
MINUTES

Figure 21. Quin2-loaded Hepatocytes Exposed to Carbon Tetrachloride or Phenylephrine

Carbon tetrachloride (2 mM) or phenylephrine (10 uM) was added to quin2-loaded hepatocytes. Fluorescence was monitored at excitation wavelength 339 nm and emission wavelength 500 nm; slit widths were 5 nm. Maximum and minimum fluorescence were determined by successive additions of digitonin (20 uM) and EGTA (4 mM), and calcium concentrations were calculated from fluorescence as described in Methods. Vertical lines indicate opening and closing of the sample compartment door for additions.


FLUORESCENCE (arbitrary units)
60
50
40
30
CCl_4
DIGITONIN
EGTA
F_{max}
1000
400
200
100
50
F_{min}
Ca_i^{++} (nM)
0
1
2
3
MINUTES


FLUORESCENCE (arbitrary units)
70
60
50
40
PHENYLEPHRINE
DIGITONIN
EGTA
F_{max}
1000
400
200
100
50
F_{min}
Ca_i^{++} (nM)
0
1
2
3
4
MINUTES

quin2 fluorescence; another halogenated hydrocarbon, 1,1-dichloroethylene, also increased quin2 fluorescence (Figure 22). By this technique, CCl_4 produced an increase in cytosolic Ca^{++} that was comparable in magnitude to that produced by phenylephrine, an agonist which acts like the Ca^{++}-mobilizing adrenergic hormones. Others have reported rapid increases in Ca^{++} to near micromolar levels after hormonal agents, as determined with quin2 (Berthon et al., 1984). The increase to approximately one uM Ca^{++} after CCl_4 was somewhat less than but comparable to that found by measurements of phosphorylase a activity in cultured hepatocytes, $\geq$3 uM Ca^{++}.

Phosphorylase a and Calcium Pump in Hepatocyte Suspensions

Experiments to estimate intracellular Ca^{++} concentrations using quin2 utilized freshly isolated hepatocyte suspensions, as distinguished from primary cultures of hepatocytes. Therefore, phosphorylase a and ER Ca^{++} pump activities were determined in hepatocyte suspensions exposed to CCl_4. Hepatocytes were maintained in Krebs-Henseleit buffer supplemented with glucose, amino acids, glutamate, fumarate, and pyruvate (see Methods) to maintain low basal phosphorylase a levels (T. Pillsworth, personal communication). Cell preparations were evaluated by stimulation of phosphorylase a activity upon exposure to 10 uM phenylephrine, and cells were discarded when they became unresponsive to phenylephrine (usually > two hours after isolation). Results in Table 10 suggest that the vehicle (0.3% ethanol) elevated phosphorylase a activity over control (no addition) levels. One mM CCl_4 did not produce more phosphorylase activation than vehicle alone, and 3 mM CCl_4 doubled phosphorylase a activity. CCl_4-induced increases in phosphorylase a activity in suspended cells (97 nmole/mg protein/minute, Table 10) never reached the

Figure 22. Quin2-loaded Hepatocytes Exposed to Phenylephrine, Carbon Tetrachloride, 1,1-Dichloroethylene, or Vehicle (Ethanol)

Phenylephrine (10 uM), carbon tetrachloride (2 mM), 1,1-dichloroethylene (4 mM), and vehicle (ethanol, 0.3% by volume) were added to different samples from a single preparation of quin2-loaded hepatocytes. Fluorescence was monitored at excitation wavelength 339 nm and emission wavelength 500 nm; slit widths were 5 nm. Maximum and minimum fluorescence were determined (not shown), and calcium concentrations were calculated from fluorescence as described in Methods. Vertical lines indicate opening and closing of the sample compartment door for additions.

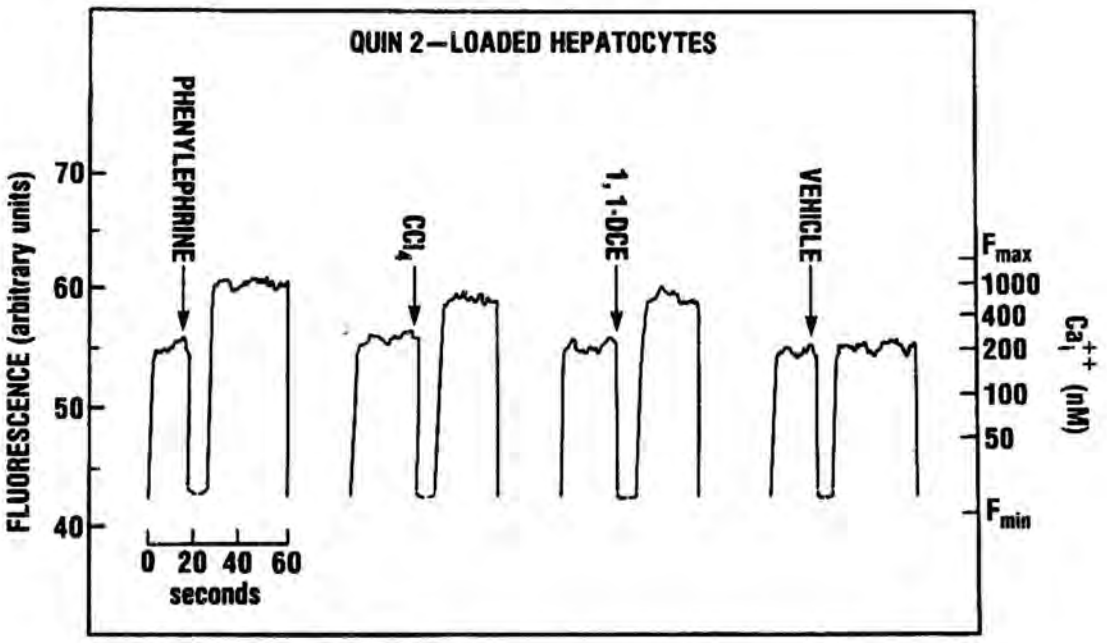

QUIN 2—LOADED HEPATOCYTES
FLUORESCENCE (arbitrary units)
70
60
50
40
PHENYLEPHRINE
CCl_4
1,1-DCE
VEHICLE
0 20 40 60
seconds
F_{max}
1000
400
200
100
50
F_{min}
Ca_i^{++} (nM)

Table 10. Phosphorylase *a* and Endoplasmic Reticulum Calcium Pump Activities in Freshly Isolated Hepatocytes Exposed to Carbon Tetrachloride

Carbon tetrachloride (1 or 3 mM) or vehicle (ethanol, 0.3% by volume) was added to suspensions of hepatocytes. Exposures were terminated at five minutes. Phosphorylase *a* activity was quantitated as nmoles inorganic phosphate released from glucose-1-phosphate/mg protein/minute, and ER calcium pump activity was determined as nmoles ^{45}Ca accumulated/mg protein/30 minutes. Data represent means $\pm$ SEM from seven cell preparations for phosphorylase *a* activity, and from three cell preparations for calcium pump activity. Differences between carbon tetrachloride and vehicle are significant at $p \leq 0.05$ (+) by least significant difference tests following one-way ANOVAs.

TABLE 10.

PHOSPHORYLASE *a* AND ENDOPLASMIC RETICULUM CALCIUM PUMP ACTIVITIES IN FRESHLY ISOLATED HEPATOCYTE SUSPENSIONS

	Phosphorylase *a* (nmol/mg prot/min)	ER Calcium Pump (nmol/mg prot/30 min)
Control (no addition)	51 ± 8	123 ± 14
Vehicle (ethanol)	78 ± 12^{+}	125 ± 7
CCl_4 (1 mM)	74 ± 11^{+}	75 ± 12^{+}
CCl_4 (3 mM)	97 ± 10^{+}	62 ± 21^{+}

levels attained with CCl_4 in cultured hepatocytes (150 to 180 nmole/mg protein/minute, Figures 12 and 13). ER Ca^{++} pump activity was unaffected by vehicle, and 1 mM and 3 mM CCl_4 significantly inhibited pump activity by 40% and 50%, respectively (Table 10).

The calibration curve obtained with cultured hepatocytes was used to estimate cytoplasmic Ca^{++} concentrations from phosphorylase a activities in hepatocyte suspensions. This assumption was believed valid because a similarly prepared calibration curve for freshly isolated suspensions of hepatocytes (from guinea pig) demonstrated comparable increases in phosphorylase a activity over the same range of Ca^{++} concentrations (Burgess et al., 1983). As estimated from phosphorylase a determinations in hepatocyte suspensions (Table 10) and the calibration curve (Figure 19), Ca^{++} rose to approximately 1 uM in cells exposed to 3 mM CCl_4 for 5 minutes. This was in good agreement with results obtained with quin2 in cell suspensions. However, phosphorylase a activity in freshly isolated hepatocytes was also responsive to 0.3% ethanol. This was thought to be unrelated to the mechanism by which CCl_4 acted, because the ER Ca^{++} pump was unaffected by the vehicle. Ethanol did not produced elevated Ca^{++} levels as determined by quin2 fluorescence in hepatocyte suspensions (Figure 22). Thus, ethanol-induced phosphorylase stimulation resulted from a Ca^{++}-independent pathway, perhaps via cyclic AMP or alternatively by a direct solvent effect on enzymes of the phosphorylase cascade. Further experiments (data not shown) suggested that dimethylsulfoxide (0.3% addition by volume) did not elevate phosphorylase a activity, and so this was used as vehicle for subsequent experiments.

Phosphorylase a after Carbon Tetrachloride or Phenylephrine

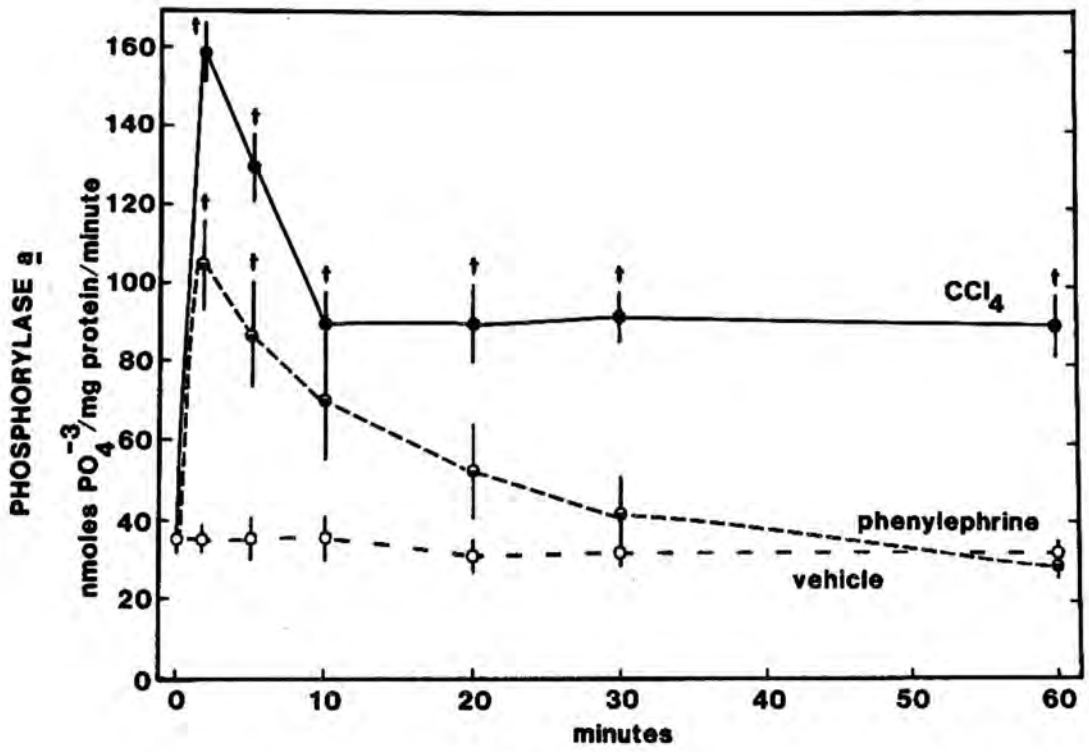
Because results obtained with quin2 suggested that CCl_4 and phenylephrine mobilized Ca^{++} to approximately the same extent, experiments were conducted to establish whether these agents differed in their durations of Ca^{++} mobilization. Cultured hepatocytes were exposed for 2.5 to 60 minutes to 10 uM phenylephrine, 3 mM CCl_4, or vehicles (1% H_2O for phenylephrine, 0.3% dimethyl sulfoxide for CCl_4). Neither vehicle had any effect on phosphorylase a activity, and those results were averaged. Figure 23 indicates that phosphorylase a stimulation following a maximally effective dose of phenylephrine was transient. Maximal phosphorylase a stimulation (3.5-fold) was achieved by 2.5 minutes, and phosphorylase a had returned essentially to basal activity by 30 minutes. In contrast, phosphorylase a activity was stimulated to a greater extent (five-fold) at 2.5 minutes following CCl_4 addition, and phosphorylase a activity remained three-fold elevated between ten to 60 minutes. Differences from basal levels were statistically significant at 2.5, 5, and 10 minutes for phenylephrine, and at 2.5 through 60 minutes for CCl_4. These results illustrate sustained activation of a Ca^{++}-stimulated enzyme following a toxic agent that is not mimicked by an alpha-adrenergic agent.

Mitochondrial and Endoplasmic Reticulum Calcium Pumps

Unlike endoplasmic reticulum Ca^{++} sequestration, mitochondrial Ca^{++} sequestration is not inhibited at early times after a single hepatotoxic dose of CCl_4 *in vivo* (Carafoli and Tiozzo, 1968, Moore *et al.*, 1976). The tremendous accumulation of Ca^{++} by mitochondria accounts in large part for the increased total liver calcium observed following CCl_4 administration to rats (Reynolds, 1964). To determine whether selective

Figure 23. Time-Dependent Effects of Carbon Tetrachloride or Phenylephrine on Phosphorylase a Activity in Cultured Hepatocytes

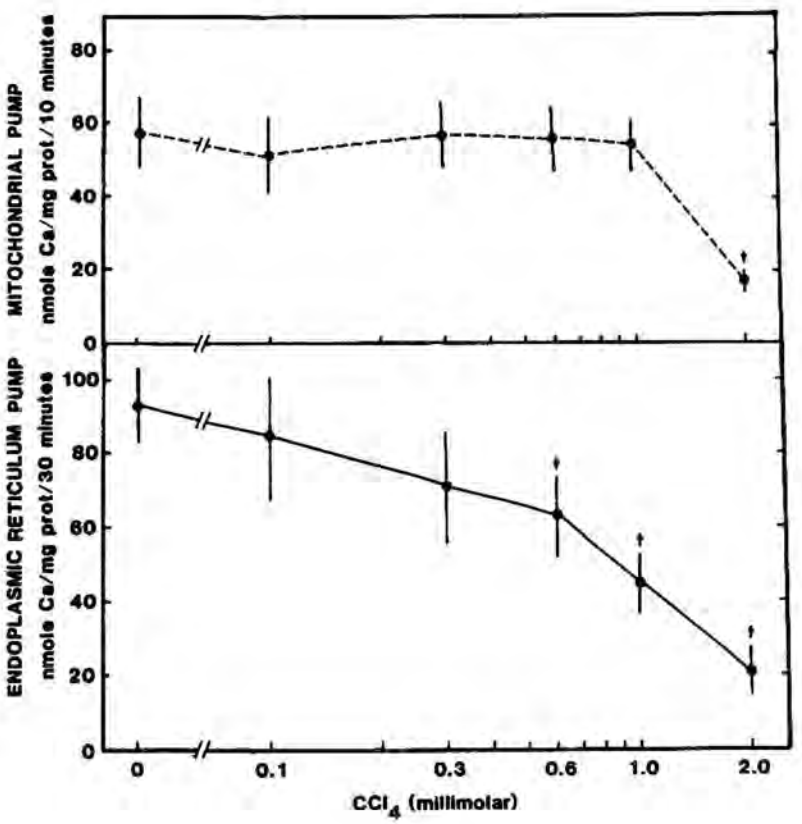
Carbon tetrachloride (3 mM), phenylephrine (10 uM), or vehicle (dimethyl sulfoxide or water) was added to hepatocyte cultures at zero minutes. Exposures were terminated at 2.5 through 30 minutes. Phosphorylase a activity was quantitated as nmoles inorganic phosphate released from glucose-1-phosphate/mg protein/minute. Data represent means $\pm$ SEM from three cell preparations. Differences between treatments and vehicle are significant at $p \leq 0.05$ (+) by least significant difference test following two-way ANOVA.


PHOSPHORYLASE a
nmoles PO_4^{-3}/mg protein/minute
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
minutes
CCl_4
phenylephrine
vehicle

inhibition by CCl_4 of Ca^{++} accumulation by these organelles occurred *in vitro*, mitochondrial and endoplasmic reticulum Ca^{++} pump activities were investigated in dose/response experiments with CCl_4. Activities were assayed in homogenates of cultured cells at conditions optimized for each Ca^{++} pump as described in Methods. Control mitochondrial Ca^{++} pump activity averaged 58 nmole/mg protein/10 minutes. Control ER Ca^{++} pump activity averaged 94 nmol/mg protein/30 minutes; this value was somewhat higher than values obtained previously. These experiments were performed with cells that were not frozen subsequent to sample collection, because mitochondrial Ca^{++} pump cannot withstand freezing under these conditions. Doses of CCl_4 between 0.1 and 2.0 mM progressively inhibited ER Ca^{++} pump activity, with half-maximal inhibition occurring at approximately 0.8 mM CCl_4 (Figure 24). In contrast, doses between 0.1 and 1.0 mM CCl_4 did not significantly affect mitochondrial Ca^{++} accumulation. Two mM CCl_4 severely decreased mitochondrial Ca^{++} pump activity to 28% of control levels. Thus, within a narrow dose range (0.1 to 1.0 mM CCl_4), endoplasmic reticulum but not mitochondrial Ca^{++} sequestration was inhibited in cultured hepatocytes exposed to CCl_4. Santone and Acosta (1985) examined Ca^{++} pumps in organelles isolated from cultured hepatocytes exposed to CCl_4, and found that inhibition of the Ca^{++} pump at endoplasmic reticulum developed at lower doses than inhibition of the mitochondrial Ca^{++} pump. Most of the experiments performed here with cultured hepatocytes employed doses of CCl_4 (2 mM and 3 mM) that likely inhibited both mitochondrial and endoplasmic reticulum Ca^{++} sequestration. As a result, it is possible that CCl_4-induced phosphorylase activation and further sequelae attributed to ER Ca^{++} pump inhibition in fact resulted from impaired Ca^{++} sequestration at more than just that

Figure 24. Dose-Dependent Effects of Carbon Tetrachloride on Mitochondrial and Endoplasmic Reticulum Calcium Pump Activities in Cultured Hepatocytes

Carbon tetrachloride (0.1 to two mM) or vehicle (dimethylsulfoxide, 0.3% by volume) was added to hepatocyte cultures. Exposures were terminated at five minutes. Calcium pump activities were assayed in hepatocyte homogenates, under conditions optimized for Ca^{++} accumulation by mitochondria or by endoplasmic reticulum vesicles, as described in Methods. Mitochondrial calcium pump activity was quantitated as nmoles ^{45}Ca accumulated/mg protein/10 minutes, and endoplasmic reticulum calcium pump activity was determined as nmoles ^{45}Ca accumulated/mg protein/30 minutes. Data represent means $\pm$ SEM from three cell preparations. Differences between carbon tetrachloride and vehicle are significant at $p \leq 0.05$ (+) by least significant difference tests following one-way ANOVAs.


MITOCHONDRIAL PUMP
nmole Ca/mg prot/10 minutes
80
60
40
20
0
ENDOPLASMIC RETICULUM PUMP
nmole Ca/mg prot/30 minutes
100
80
60
40
20
0
0
0.1
0.3
0.6
1.0
2.0
CCl4 (millimolar)

organelle. In order to best model CCl_4 hepatotoxicity *in vivo*, future studies should use doses of CCl_4 between 0.1 and 1.0 mM.

DISCUSSION

EVIDENCE THAT CARBON TETRACHLORIDE INCREASES CYTOSOLIC CALCIUM

Activation of glycogen phosphorylase to the a form, independent of changes in cAMP levels, is a useful "endogenous indicator" of cytosolic Ca^{++} concentrations in vivo (Keppens and DeWulf, 1975, 1976) and in isolated hepatocytes (Keppens et al., 1977, Murphy et al., 1980). Phosphorylase a activity has been used to follow cytoplasmic Ca^{++} in hepatocyte suspensions exposed to the toxic agents tert-butyl hydroperoxide (Bellomo et al., 1984) and acetaminophen (Moore et al., 1985). Results presented here are the first reported application of this technique to determination of changes in hepatic cytosolic Ca^{++} levels produced by CCl_4. Elevated cytoplasmic Ca^{++} levels were determined by glycogen phosphorylase a activity in liver from intact rats and in cultured rat hepatocytes. Changes in cytosolic Ca^{++} were confirmed using the fluorescent indicator quin2 in suspensions of hepatocytes exposed to CCl_4.

In these experiments, increased phosphorylase a activity suggested that cytoplasmic Ca^{++} concentrations rapidly became elevated in rat liver (within 30 minutes) and in cultured hepatocytes (within 2.5 minutes) exposed to CCl_4 (Figures 4 and 12). Elevated phosphorylase a levels were sustained in liver tissue ($\geq$24 hours) and in isolated cells ($\geq$30 minutes). Increased phosphorylase a activity was interpreted to reflect increased cytosolic Ca^{++} concentrations because no detectable increases in cellular cAMP were found at early times in vivo or in hepatocytes (Table 2 and Figure 17). Phosphorylase a activity, and thus Ca^{++} concentrations, increased concomitantly with inhibition of the ER Ca^{++} pump in time course studies with CCl_4 in vivo and in cultured

hepatocytes (Figures 4 and 12).

In dose/response studies with cultured hepatocytes, ER Ca^{++} pump activity had to be inhibited by greater than 50% before phosphorylase was converted to the a form (Figure 13). Perhaps there is "reserve" Ca^{++} sequestering ability so that moderate insults to the endoplasmic reticulum do not compromise Ca^{++} homeostasis in the liver cell. Or, other intracellular Ca^{++} controlling mechanisms (mitochondria, plasma membrane) may also become inhibited at the higher doses of CCl_4. Loss of Ca^{++} extruding/sequestering functions at other organelles could contribute directly to increased cytosolic Ca^{++}, or could contribute indirectly by no longer compensating for the loss of endoplasmic reticulum function. Extracellular Ca^{++} was not required for phosphorylase activation at early times in cultured hepatocytes (Table 9). This suggests that CCl_4 causes release of Ca^{++} from intracellular stores, and that this release is sufficient to elevate cytosolic Ca^{++} concentrations to levels that can activate Ca^{++}-responsive enzymes such as glycogen phosphorylase a. Others have reported that Ca^{++} is selectively lost from microsomal (but not mitochondrial) fractions derived from rats (Reynolds, 1972, Moore, 1981, Kroner, 1982) or from hepatocytes (Ray and Moore, 1986) exposed to CCl_4, implying that endoplasmic reticulum is the source of Ca^{++} released intracellularly.

CCl_4 exposure produced considerable hepatic cell death, as indicated by release of glutamic-pyruvic transaminase, at late times *in vivo* (eight and 24 hours), and at late times (15 minutes through two hours) in hepatocytes (Figures 9 and 16). In cultured cells, glutamic-pyruvic transaminase release increased progressively between 1 and 4 mM CCl_4 (Figure 16), although inhibition of ER Ca^{++} pump activity and stimula-

tion of phosphorylase a activity were maximal by 2 mM CCl_4. Possibly this hepatotoxic response was progressive because Ca^{++} concentrations were continually increasing to higher levels than elevated phosphorylase a activity could report. An influx of extracellular Ca^{++} after 15 minutes at 3 or 4 mM CCl_4 may have accelerated this effect. At these high doses, other mechanisms of CCl_4 toxicity (lipid peroxidation, covalent binding to critical macromolecules) could also have been occurring. Loss of cytoplasmic enzyme always followed sustained phosphorylase activation *in vivo* and in cultured hepatocytes. Thus, doses of CCl_4 that produced elevated phosphorylase a levels could be correlated with later evidence of liver cell damage. This is consistent with the hypothesis that elevated cytosolic Ca^{++} concentrations could be causally related to CCl_4 hepatotoxicity.

Many physiologic agents elevate cytoplasmic Ca^{++} levels, and it was essential to distinguish a Ca^{++}-mobilizing non-hepatotoxin from the hepatotoxin CCl_4. The time-dependent effects of phenylephrine and CCl_4 on phosphorylase a activity were contrasted in cultured hepatocytes (Figure 23). CCl_4 exposure produced elevated phosphorylase a levels that were sustained for $\geq$60 minutes, while phosphorylase a returned to basal levels within ten minutes after phenylephrine. Thus, CCl_4 caused prolonged stimulation of a Ca^{++}-responsive enzyme that was not mimicked by a hormone-like agent. CCl_4 also stimulated phosphorylase a activity to a greater extent than did phenylephrine. Phenylephrine did not produce greater stimulation of phosphorylase a activity either at higher doses (>10 uM) or at earlier times (one or two minutes) than used here (R. M. Long, unpublished). Production of intracellular Ca^{++} concentrations that are supraphysiologic, either in duration or magnitude, in

liver cells exposed to CCl_4 could explain why CCl_4 is hepatotoxic and phenylephrine is not. In Figure 23, the curves for phosphorylase a activity may reflect Ca^{++} concentrations directly, or they may represent the state of activation of Ca^{++}-stimulated enzymes. For example, one possible interpretation following CCl_4 exposure is that during the initial phase (0 to 2.5 minutes), cytoplasmic Ca^{++} concentrations are rapidly rising due to release of Ca^{++} from the endoplasmic reticulum as a consequence of ER Ca^{++} pump inhibition. During the next phase (2.5 to 5 minutes), cytoplasmic Ca^{++} concentrations are declining, perhaps as a result of extrusion of Ca^{++} by the plasma membrane Ca^{++} pump or accumulation of Ca^{++} by mitochondria. Then by the final phase (10 to 60 minutes), cytoplasmic Ca^{++} concentrations remain at a constant, elevated level as cells have lost their ability both to extrude and to sequester Ca^{++}. An alternative explanation would be that progressively increasing Ca^{++} concentrations may have differential effects on Ca^{++}-responsive enzymes. For example, moderate increases in cytoplasmic Ca^{++} may stimulate enzyme activities (including kinases and phosphatases that determine the net activation state of phosphorylase), while very high Ca^{++} concentrations may inhibit the activities of the same enzymes.

In CCl_4-treated cultured hepatocytes, elevated phosphorylase a activities predicted that intracellular Ca^{++} concentrations rose to ≥ 3 uM (Figures 13 and 19). Cytosolic Ca^{++} levels on the order of one uM were obtained with the fluorescent Ca^{++} indicator compound quin2 in hepatocyte suspensions exposed to CCl_4 (Figure 21 and Results). The rise in cytoplasmic Ca^{++} occurred within 20 seconds in quin2 studies, and in ≤ 2.5 minutes (the earliest time examined) in phosphorylase studies. Phosphorylase a determinations in suspensions of hepatocytes

confirmed that cultured and freshly isolated rat hepatocytes did not differ greatly in their responses to higher doses (two to three mM) of CCl_4 (Table 10). Estimates of cytoplasmic Ca^{++} both from phosphorylase a activity and from quin2 fluorescence predicted that CCl_4 exposure elevated Ca^{++} to at least micromolar levels in liver cells. Thus, results obtained by these two methods were in agreement. With both of these techniques, maximal Ca^{++} concentrations that could be determined by either methodology were found. Cytosolic Ca^{++} could have reached much higher levels that were not detected by either of these methods. Another indicator compound that possibly could be employed to look at higher Ca^{++} levels is arsenazo III, a metallochromic compound that binds Ca^{++} less avidly than quin2. Arsenazo III does not enter cells and must be used extracellularly by a "null-point titration" technique, as described by Murphy et al. (1980).

COMPARISON OF CULTURED RAT HEPATOCYTES WITH RAT LIVER IN VIVO

Isolated rat liver cells provide an attractive model for investigations of hepatic function. Hepatocytes maintained in culture provide the advantages over freshly isolated cells of repaired plasma membranes, replenished ATP stores, improved hormonal responsiveness, and consistently high viabilities that do not decline over several hours (Bissell and Guzelian, 1980, Bissell et al., 1973). Plasma membrane integrity is an important consideration for examination of intracellular Ca^{++} concentrations, because of the normal 10,000-fold gradient of extra- to intracellular Ca^{++}. Additionally, healthy and stable cell preparations are desirable for use in toxicity studies, because dying cells present in hepatocyte suspensions complicate investigations of cell injury and death. Many aspects of CCl_4 hepatotoxicity in vivo have been reproduced

in experiments with hepatocyte preparations, including lipid peroxidation (Gravela et al., 1979, Poli et al., 1979), covalent binding of fragments derived from CCl_4 to cellular macromolecules (Casini and Farber, 1981, Chenery et al., 1981), inhibition of glucose-6-phosphatase (Poli et al., 1981), inhibition of the ER Ca^{++} pump (Pencil et al., 1982), and inhibition of lipoprotein secretion (Pencil et al., 1984).

In order to further assess the value of isolated cells as a model for liver tissue in toxicity studies, experiments here characterized the appearance of elevated phosphorylase a activity and other alterations in hepatic enzyme activities produced by CCl_4 both in vivo and in vitro. Table 7 summarizes time-dependent changes in hepatic enzyme activities in liver from intact rats and in cultured hepatocytes exposed to CCl_4. Following an inital phase for absorption in vivo (15 minutes), qualitative trends in the data appear quite similar between the two models. In rat liver and in hepatocytes, inhibition of the ER Ca^{++} pump and stimulation of phosphorylase a activity developed simultaneously. Glycogen depletion as a result of sustained phosphorylase activation by CCl_4 was demonstrated in vivo (Figure 5). Although not demonstrated here, glycogen depletion following CCl_4 exposure has also been confirmed in rat hepatocytes by Dolak et al. (1983). In vivo and in vitro, inhibition of the endoplasmic reticulum enzyme glucose-6-phosphatase developed slowly and to a lesser extent than did ER Ca^{++} pump inhibition. This indicated that the Ca^{++} pump was particularly susceptible to the actions of CCl_4. The ER Ca^{++} pump and glucose-6-phosphatase could be differently distributed about the endoplasmic reticulum; the Ca^{++} pump could be in closer proximity to the site of generation of the reactive metabolites(s) of CCl_4 by cytochrome P-450. Alternatively, cells located in the regions

of liver first injured following CCl_4 treatment could be relatively enriched in ER Ca^{++} pump rather than glucose-6-phosphatase. In liver and in hepatocytes, 5′-nucleotidase was never significantly affected by CCl_4 exposure. This finding could have implied that some of the biochemical functions of plasma membranes of liver cells were not grossly altered by CCl_4 treatment in either model. In contrast, others have reported that CCl_4 given *in vivo* alters hepatic plasma membrane permeability (Tsokos-Kuhn *et al.*, 1986). Finally, the cytoplasmic enzyme glutamic-pyruvic transaminase was released from liver cells into blood or from hepatocytes into culture medium only after the ER Ca^{++} pump was inhibited and phosphorylase *a* activity was elevated for prolonged periods ($\geq$8 hours *in vivo*, $\geq$15 minutes in hepatocytes). This would allow time for sustained elevations of Ca^{++} to initiate cellular changes leading to hepatotoxicity.

Although CCl_4-induced characteristic changes in enzyme activities evidently followed the same continuum in both models, changes occurred much more rapidly in cultured hepatocytes (within 30 minutes) than *in vivo* (within eight hours). It is not known why development of toxicity was so fast *in vitro*. One possible explanation is that liver cells in the intact tissue experience changing halocarbon concentrations as blood carrying CCl_4 flows through the organ and metabolites are cleared, thus liver is exposed only briefly to CCl_4. Glende and Pushpendran estimated that portal vein blood levels after a moderate intragastric dose of CCl_4 (0.6 to 2.5 ml/kg) attained a few tenths millimolar. Concentrations of CCl_4 added to cultured hepatocytes for most of these studies (2 and 3 mM) may have been somewhat excessive. However, because culture dishes are not sealed containers, effective concentrations of CCl_4 could have

been much lower after a very short time. Phosphorylase *a* activity was stimulated to a greater extent at early times in hepatocytes (four-fold at 5 minutes) than *in vivo* (1.4-fold at 30 minutes) in these studies. This may have initiated the speedier development of toxicity in isolated cells. Both mitochondrial and ER Ca^{++} pumps were inhibited in hepatocytes exposed to $\geq$2 mM CCl_4, a situation thought not to occur *in vivo* (Carafoli and Tiozzo, 1968). Optimally, lower concentrations of CCl_4 (1 mM or less) may need to be employed for longer time course studies. Perhaps hepatocytes in culture were injured rapidly because impaired mitochondrial functions (loss of this Ca^{++} sequestering mechanism, declining energy [ATP] stores) accelerated CCl_4 toxicity. Tyson *et al.* (1983) obtained similar results when they attempted to correlate *in vivo* and *in vitro* hepatotoxicity for a series of structurally similar haloalkanes. Evidence of toxicity, monitored as release of cytoplasmic enzymes, occurred at earlier times with rat hepatocyte suspensions (30 minutes to two hours) than with intact rats (24 to 48 hours). They compared the toxic potencies of the compounds and found the same rank orders *in vivo* and *in vitro*, as was found here for bromotrichloromethane and chloroform.

A prerequisite for CCl_4 hepatotoxicity *in vivo* that was considered here was dependence of toxicity upon metabolism by cytochrome P-450 (reviewed by MacDonald, 1983). A disadvantage with the use of cultured hepatocytes is loss of 50% or more of cytochrome P-450 content within the first 24 hours (Bissell and Guzelian, 1980). In cultured hepatocytes used for these studies, total cytochrome P-450 content was almost halved after 18 hours in culture (Table 6). When phosphorylase activation and ER Ca^{++} pump inhibition were evaluated in cells maintained on

ice, the actions of CCl_4 were essentially prevented (Table 8). Protection afforded at 0-4° C could have resulted from blocked metabolism involving cytochrome P-450. Attempts were made to manipulate the same parameters in dose/response experiments with hepatocytes using cytochrome P-450 inhibitors added *in vitro* or cytochrome P-450 inducers given *in vivo*. Anticipated shifts in the dose/response curves were not observed (data not shown). Either CCl_4's effects on the ER Ca^{++} pump and phosphorylase *a* activity did not require metabolic activation in cultured hepatocytes, or dependence upon metabolism could not be demonstrated in this system. Others have reported lack of success relating various toxic effects of CCl_4 to metabolism by cytochrome P-450 in both hepatocyte cultures (Stacey and Priestly, 1978a) and fresh hepatocyte suspensions (Gravela *et al.*, 1979, Cunningham *et al.*, 1981). In this respect, isolated hepatocytes may not entirely appropriately model the hepatotoxicity of CCl_4 *in vivo*. Or perhaps excessive doses of CCl_4 were used, and dependence upon metabolism could be demonstrated if lower doses of CCl_4 were used for longer times in hepatocyte cultures.

SIGNIFICANCE OF ELEVATED HEPATIC CYTOSOLIC CALCIUM

This work establishes that in liver cells exposed to CCl_4 *in vivo* and *in vitro*, cytosolic Ca^{++} concentrations rapidly become elevated and are sustained at micromolar levels. As a consequence, elevated cytoplasmic Ca^{++} potentially could overstimulate a variety of cell regulatory (*e.g.* kinases, phosphatases, cyclases, phosphodiesterases) and hydrolytic (*e.g.* phospholipases, proteases, nucleases) enzymes. Phospholipases A_2 and C, in particular, are plausible candidates for mediators of cellular injury, because they can directly attack membranes (*e.g.* lysosomal or plasma membranes), and they can also release free

fatty acids capable of modulating membrane functions and permeability. Exogenous application of phospholipases, but not proteases or nucleases, is toxic to cells (reviewed by Shier, 1982). Activation of phospholipases has been implicated in liver ischemic injury (Chien et al., 1978) and in Ca^{++}-dependent killing of Swiss 3T3 fibroblasts (Shier and DuBourdieu, 1982). A role for proteases has been suggested in cystamine-induced injury of hepatocytes, because protease inhibitors protect from toxicity (Orrenius, 1986). Endonucleases appear to be important for cell death that occurs during the normal course of embryologic development (Wyllie, 1986). In contrast, Toyo-oka et al. (1985) could not establish that phospholipases or proteases were involved in ischemic myocardial cell injury in studies using inhibitors of those enzymes.

Glende and Pushpendran (1986) have demonstrated that phospholipase A_2 activity is 1.4- to 5.3-fold stimulated in suspensions of rat hepatocytes exposed for 30 to 60 minutes to somewhat lower concentrations of CCl_4 than used in these studies. Lamb et al. (1984) reported that phospholipase C is rapidly activated in cultured hepatocytes (one to five minutes) and in liver from rats (30 minutes to one hour) exposed to moderate doses of CCl_4. However, others reported that they did not find phospholipase C activation after CCl_4 (Glende and Pushpendran, 1986). Camacho and Rubalcava (1984) found that total phospholipid content of rat liver plasma membranes is decreased by 60% within one day after CCl_4 administration in vivo. Accelerated phospholipid turnover and resultant breakdown of plasma membrane integrity could represent the important link between elevated intracellular Ca^{++} concentrations and later toxic events such as the eventual loss of cytoplasmic enzymes from cells exposed to CCl_4. At this time, changes in the activities of proteases

and nucleases have not yet been investigated in liver cells exposed to CCl_4.

Hepatotoxicity caused by CCl_4 probably cannot be exclusively linked to any single cellular event, particularly because this toxicant produces a myriad of effects. More likely, a complex of concurrent toxic events takes place. It could be envisioned that an initial elevation of cytosolic Ca^{++} causes perturbations leading to changes in membrane fluidity, intracellular ion or osmotic balances, energy stores, or cellular turnover and repair processes. Compromised cells may become susceptible to any of the other non-Ca^{++}-mediated actions of CCl_4, such as lipid peroxidative damage. In this regard, it might be simpler to investigate the role of elevated cytosolic Ca^{++} in toxicity using a compound that is more selective in its effects. For example, 1,1-dichloroethylene inhibits the ER Ca^{++} pump but does not produce the lipid peroxidation associated with CCl_4 (Jaeger et al., 1973, Moore, 1980).

In summary, cytoplasmic Ca^{++} levels rise rapidly and elevated Ca^{++} concentrations are sustained, concomitant with inhibition of the ER Ca^{++} pump in liver tissue or hepatocytes exposed to CCl_4. Early disruptions in hepatic Ca^{++} homeostasis are selective and precede observations of cellular injury in vivo and in vitro. It is concluded that cytosolic Ca^{++} becomes elevated with a time course that would allow Ca^{++} to play a role in CCl_4-induced hepatotoxicity. Perhaps supraphysiologic intracellular Ca^{++} levels stimulate excessive activation of Ca^{++}-sensitive enzymes capable of initiating irreversible liver cell injury.

REFERENCES

Acosta, D., D. C. Anuforo, and R. V. Smith. (1980) Cytotoxicity of Acetaminophen and Papaverine in Primary Cultures of Rat Hepatocytes. Toxicol. Appl. Pharmacol. 53:306-314.

Acosta, D., and E. M. B. Sorenson. (1983) Role of Calcium in Cytotoxic Injury of Cultured Hepatocytes. Ann. NY Acad. Sci. 407:78-92.

Armitage, P. (1971) Statistical Methods in Medical Research. Blackwell Scientific Publications, Boston, MA.

Akerman, K. E. O. (1982) Calcium Transport and Cell Activation. Med. Biol. 60:168-182.

Alvares, A. P., and A. Kappas. (1977) Heterogeneity of Cytochrome P-450s Induced by Polychlorinated Biphenyls. J. Biol. Chem. 252:6373-6378.

Aronson, N. N., and O. Touster. (1974) Isolation of Rat Liver Plasma Membrane Fragments in Isotonic Sucrose. In: Methods in Enzymology, Vol. 31, ed. S. Fleisher and L. Packer. Academic Press, NY, 90-102.

Assimacopoulos-Jeannet, F. D., P. F. Blackmore, and J. H. Exton. (1977) Studies on the alpha-Adrenergic Activation of Hepatic Glucose Output. J. Biol. Chem. 252:2662-2669.

Bellomo, G. H., H. Thor, and S. Orrenius. (1984) Increase in Cytosolic Calcium Concentrations during t-butyl hydroperoxide Metabolism by Isolated Hepatocytes Involves NADPH Oxidation and Mobilization of Intracellular Calcium Stores. FEBS Lett. 168:38-42.

Bernstein, J., and G. Santacana. (1985) The Sodium/Calcium Exchange System of the Liver Cell. Res. Commun. Chem. Pathol. Pharmacol. 47:3-34.

Berry, M. N., and D. S. Friend. (1969) High Yield Preparation of Isolated Rat Liver Parenchymal Cells. J. Cell Biol. 43:506-520.

Bers, D. M. (1982) A Single Method for the Accurate Determination of Free Calcium Concentrations in Calcium-EGTA Solutions. Am. J. Physiol. 242:C404-C408.

Berthon, B., A. Binet, J.-P. Mauger, and M. Claret. (1984) Cytosolic Free Calcium in Isolated Rat Hepatocytes as Measured by Quin2. FEBS Lett. 167:19-24.

Binet, A., B. Berthon, and M. Claret. (1985) Hormone-Induced Increase in Free Cytosolic Calcium and Glycogen Phosphorylase Activation in Rat Hepatocytes Incubated in Normal and Low Calcium Media. Biochem. J. 228:565-574.

Bissell, D. M., L. E. Hammaker, and U. A. Meyer. (1973) Parenchymal Cells from Adult Rat Liver in Nonproliferating Monolayer Culture. I. Functional Studies. J. Cell Biol. 59:722-734.

Bissell, D. M., and P. S. Guzelian. (1980) Phenotypic Stability of Adult Rat Hepatocytes in Primary Monolayer Culture. Ann. NY Acad. Sci. 349:85-98.

Bradford, M. M. (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein Dye Binding. Anal. Biochem. 72:248-254.

Brostrom, C. O., and C. Kon. (1974) An Improved Protein Binding Assay for Cyclic AMP. Anal. Biochem. 58:459-468.

Burgess, G. M., P. P. Godfrey, J. S. McKinney, M. J. Berridge, R. F. Irvine, and J. W. Putney, Jr. (1984) The Second Messenger Linking Receptor Activation to Internal Calcium Release in Liver. Nature 309:63-66.

Burgess, G. M., J. S. McKinney, A. Fabiato, B. A. Leslie, and J. W. Putney, Jr. (1983) Calcium Pools in Saponin-Permeabilized Guinea Pig Hepatocytes. J. Biol. Chem. 258:15336-15345.

Camacho, J. and B. Rubalcava. (1984) Lipid Composition of Liver Plasma Membranes From Rats Intoxicated With Carbon Tetrachloride. Biochim. Biophys. Acta 776:97-104.

Carafoli, E. (1967) In Vivo Effects of Uncoupling Agents in the Incorporation of Calcium and Strontium into Mitochondria and Other Subcellular Fractions of Rat Liver. J. Gen. Physiol. 50:1849-1864.

Carafoli, E. (1982) Membrane Transport and the Regulation of the Cell Calcium Levels. In: Pathophysiology of Shock, Anoxia, and Ischemia, ed. R. A. Cowley and B. F. Trump. Williams and Wilkins, Baltimore, MD, 95-112.

Carafoli, E. and R. Tiozzo. (1968) A Study of Energy-Linked Calcium Transport in Liver Mitochondria During Carbon Tetrachloride Intoxication. Exp. Mol. Pathol. 9:131-140.

Carlson, G. P. (1975) Potentiation of Carbon Tetrachloride Hepatotoxicity in Rats by Pretreatment with Polychlorinated Biphenyls. Toxicology 6:69-77.

Casini, A. F., and J. L. Farber. (1981) Dependence of the Carbon Tetrachloride-Induced Death of Cultured Hepatocytes on the Extracellular Calcium Concentration. Am. J. Pathol. 105:138-148.

Castro, J. A., and M. I. Diaz-Gomez. (1972) Studies on the Irreversible Binding of [^{14}C]-Carbon Tetrachloride to Microsomal Lipids in Rats under Varying Experimental Conditions. Toxicol. Appl. Pharmacol. 23:541-552.

Chapman, G. S., A. L. Jones, U. A. Meyer, and D. M. Bissell. (1973) Parenchymal Cells from Adult Rat Liver in Nonproliferating Monolayer Culture. J. Cell Biol. 59:735-747.

Charest, R., P. F. Blackmore, B. Berthon, and J. H. Exton. (1983) Changes in Free Cytosolic Calcium in Hepatocytes Following alpha-Adrenergic Stimulation. J. Biol. Chem. 258:8769-8773.

Chenery, R., M. George, and G. Krishna. (1981) The Effect of Ionophore A23187 and Calcium on Carbon Tetrachloride-Induced Toxicity in Cultured Hepatocytes. Toxicol. Appl. Pharmacol. 60:241-252.

Chien, K. R., J. Adams, A. Serroni, J. T. Martin, J. L. Farber. (1978) Accelerated Phospholipid Degradation and Associated Membrane Dysfunction in Irreversible, Ischemic Liver Cell Injury. J. Biol. Chem. 253:4809-4817.

Cunningham, M. L., A. J. Gandolfi, K. Brendel, and I. G. Sipes. (1981) Covalent Binding of Halogenated Volatile Solvents to Subcellular Macromolecules in Hepatocytes. Life Sci. 29:1207-1212.

Diaz-Gomez, M. I., C. R. deCastro, N. D'Acosta, O. M. deFenos, E. C. deFerreyra, and J. A. Castro. (1975) Species Differences in Carbon Tetrachloride-Induced Hepatotoxicity: The Role of Carbon Tetrachloride Activation and of Lipid Peroxidation. Toxicol. Appl. Pharmacol. 34:102-114.

Dolak, J. A., P. Y. Lee, W. J. Brattin, and R. O. Recknagel. (1983) Effect of Carbon Tetrachloride on the Glycogen Content of Isolated Hepatocytes. Fed. Proc. 42:513.

Exton, J. H., G. A. Robinson, E. W. Sutherland, and C. R. Park. (1971) Studies on the Role of Adenosine 3',5'-Monophosphate in the Hepatic Actions of Glucagon and Catecholamines. J. Biol. Chem. 246:6166-6177.

Fahl, W. E., G. Michalopoulos, G. L. Sattler, C. R. Jefcoate, and H. C. Pitot. (1979) Characteristics of Microsomal Enzyme Controls in Primary Cultures of Rat Hepatocytes. Arch. Biochem. Biophys. 192:61-72.

Farber, J. L. (1980) Reactions of the Liver to Injury. In: Toxic Injury of the Liver, Part A, ed. E. Farber and M. M. Fischer. Marcel Dekker, Inc., NY, 215-241.

Fander, U., W. Haas, and H. Kroner. (1982) The Damage of the Hepatic Mixed Function Oxygenase System by Carbon Tetrachloride: Significance of Incorporation of [^{14}C]-Carbon Tetrachloride Metabolites In Vivo. Exp. Mol. Pathol. 36:34-43

Fariss, M. W., and D. J. Reed. (1985) Mechanism of Chemically-Induced Toxicity. II. Role of Extracellular Calcium. Toxicol. Appl. Pharmacol. 79:296-306.

Fernandez, G., M. C. Villarrud, E. D. G. deToranzo, and J. A. Castro. (1982) Covalent Binding of Carbon Tetrachloride Metabolites to the Heme Moiety of Cytochrome P450 and its Degradation Products. Res. Commun. Chem. Pathol. Pharmacol. 35:283-290.

Fiske, C. H., and Y. Subbarow. (1925) The Colorimetric Determination of Phosphorus. J. Biol. Chem. 66:375-400.

Garner, R. C., and A. E. M. McLean. (1969) Increased Susceptibility to Carbon Tetrachloride Poisoning in Rat after Pretreatment with Phenobarbitone. Biochem. Pharmacol. 18:645-650.

Gilman, A. G., and F. Murad. (1974) Assay of Cyclic Nucleotides by Receptor Protein Binding Displacement. In: Methods in Enzymology, Vol. 38, ed. J. G. Hardman and B. W. O'Malley. Academic Press, NY, 49-61.

Glende, Jr., E. A., and C. K. Pushpendran. (1986) Activation of Phospholipase A2 By Carbon Tetrachloride in Isolated Rat Hepatocytes. Biochem. Pharmacol. In Press.

Golden, S., P. A. Wals, and J. Katz. (1977) An improved Procedure for the Assay of Glycogen Synthetase and Phosphorylase in Rat Liver Homogenates. Anal. Biochem. 77:436-445.

Good, H., H. Kramer, and M. Somogyi. (1933) The Determination of Glycogen. J. Biol. Chem. 100:485-491.

Grant, M. H., M. A. L. Melvin, P. Shaw, W. T. Melvin, and M. D. Burke. (1985) Studies on the Maintenance of Cytochromes P-450 and b_5 Monooxygenases and Cytochrome Reductases in Primary Cultures of Hepatocytes. FEBS Lett. 190:99-103.

Gravela, E., E. Albano, M. U. Dianzani, G. Poli, and T. F. Slater. (1979) Effects of Carbon Tetrachloride on Isolated Rat Hepatocytes: Inhibiiton of Protein and Lipoprotein Secretion. Biochem. J. 178:509-512.

Guzelian, P. S., D. M. Bissell, and U. A Meyer. (1977) Drug Metabolism in Adult Rat Hepatocytes in Primary Monolayer Culture. Gastroenterology 72:1232-1239.

Head, B., D. E. Moody, C. H. Woo, and E. A. Smuckler. (1981) Alterations of Specific Forms of Cytochrome P-450 in Rat Liver during Acute Carbon Tetrachloride Intoxication. Toxicol. Appl. Pharmacol. 61:286-295.

Hems, D. A., and P. D. Whitton. (1980) Control of Hepatic Glycogenolysis. Physiol. Rev. 60:1-50.

Hue, L., F. Bontemps, and H.-G. Hers. (1975) The Effect of Glucose and Potassium Ions on the Interconversion of the Two Forms of Glycogen Phosphorylase and of Glycogen Synthetase in Isolated Rat Liver

Preparations. Biochem. J. 152:105-114.

Huijing, F. (1970) A Rapid Enzymatic Method for Glycogen Estimation in Very Small Tissue Samples. Clin. Chim. Acta 30:567-572.

Ishikawa, K., and T. Shimazu. (1976) Daily Rhythms of Glycogen Synthetase and Phosphorylase Activities in Rat Liver: Influences of Food and Light. Life Sci. 19:1873-1878.

Jaeger, R. J., M. J. Trabulus, and S. D. Murphy. (1973) Biochemical Effects of 1,1-Dichloroethylene in Rats: Dissociation of Its Hepatotoxicity from a Lipoperoxidative Mechanism. Toxicol. Appl. Pharmacol. 24:457-467.

Jernigan, J. D., and R. D. Harbison. (1982) Role of Biotransformation in the Potentiation of Halocarbon-Induced Hepatotoxicity by 2,5-Hexanedione. J. Toxicol. Environ. Health 9:761-781.

Jernigan, J. D., J. G. Pounds, and R. D. Harbison. (1983) Potentiation of Chlorinated Hydrocarbon Toxicity by 2,5-Hexanedione in Primary Cultures of Adult Rat Hepatocytes. Fundam. Appl. Toxicol. 3:22-26.

Joseph, S. K., K. E. Coll, A. P. Thomas, R. Rubin, and J. R. Williamson. (1985) The Role of Extracellular Calcium in the Response of the Hepatocyte to Calcium-Dependent Hormones. J. Biol. Chem. 260:12508-12515.

Joseph, S. K., A. P. Thomas, R. J. Williams, R. F. Irvine, and J. R. Williamson. (1984) Myo-Inositol 1,4,5-Trisphosphate. J. Biol. Chem. 259:3077-3081.

Judah, J. D., K. Ahmed, A. E. McLean. (1964) Possible Role of Ion Shifts in Liver Injury. In: CIBA Symposium on Cellular Injury, ed. A. V. S. deReuck and J. Knight. J. London and A. Churchill, Ltd., London, 187-205.

Keppens, S., and H. deWulf. (1975) The Activation of Liver Glycogen Phosphorylase by Vasopressin. FEBS Lett. 51:29-32.

Keppens, S., and H. deWulf. (1976) The Activation of Liver Glycogen Phosphorylase by Angiotensin II. FEBS Lett. 68:279-282.

Keppens, S., J. R. Vandenheede, and H. deWulf. (1977) On the Role of Calcium as Second Messenger in Liver for the Hormonally Induced Activation of Glycogen Phosphorylase. Biochim. Biophys. Acta 496:448-457.

Klaassen, C.D., and G. L. Plaa. (1969) Comparison of the Biochemical Alterations Elicited in Livers From Rats Treated with Carbon Tetrachloride, Chloroform, 1,1,2-Trichloroethylene, and 1,1,1-Trichloroethylene. Biochem. Pharmacol. 18:2019-2027.

Kroner, H. (1982) The Intracelluar Distribution of Liver Cell Calcium in Normal Rats and One Hour after Administration of Carbon Tetrachloride. Biochem. Pharmacol. 31:1069-1073.

Krstulovic, A. M., and P. R. Brown (1982) Reversed-Phase High-Performance Liquid Chromatography. John Wiley and Sons, New York, NY.

Lamb, R. G., S. B. McCue, D. R. Taylor, and M. A. McGuffin. (1984) The Role of Phospholipid Metabolism in Bromobenzene- and Carbon Tetrachloride-Dependent Hepatocyte Injury. Toxicol. Appl. Pharmacol. 75:510-520.

Lehninger, A. L., E. Carafoli, and C. S. Rossi. (1967) Energy-Linked Ion Movements in Mitochondrial Systems. Adv. Enzymology 29:259-320.

Lowry, O. H., N.J. Rosebrough, A. L. Farr, and R. J. Randall. (1951) Protein Determination with Folin Phenol Reagent. J. Biol. Chem. 193:265-275.

Lowry, O. N., and J. A. Lopez. (1945) The Determination of Inorganic Phosphate in the Presence of Labile Phosphate Esters. J. Biol. Chem. 162:421-428.

Lowrey, K. E., E. A. Glende, Jr., and R. O. Recknagel. (1981) Rapid Depression of Rat Liver Microsomal Calcium Pump Activity after Administration of Carbon Tetrachloride or Bromotrichloromethane, and Lack of Effect of Ethanol. Toxicol. Appl. Pharmacol. 51:389-384.

MacDonald, T. L. (1983) Chemical Mechanisms of Halocarbon Metabolism. CRC Crit. Rev. Toxicol. 11:85-120.

MacManus, J. P., D. J. Franks, T. Youdale, and B. M. Braceland. (1972) Increases in Rat Liver Cyclic AMP Concentrations Prior to the Initiation of DNA Synthesis Following Partial Hepatectomy or Hormone Infusion. Biochem. Biophys. Res. Commun. 49:1201-1207.

Magee, D. N. (1966) Toxic Liver Necrosis. Lab. Invest. 15:111-131.

Malencik, D. A., and E. H. Fischer. (1982) Structure, Function, and Regulation of Phosphorylase Kinase. In: Calcium and Cell Function, ed. W. Y. Cheung. Academic Press, NY, 161-188.

Martell, A. E., and R. M. Smith. (1974) Critical Stability Constants, Vol 1. Plenum Press, NY, 269.

Mitchell, J. R., D. J. Jollow, W. Z. Potter, D. C. Davis, J. R. Gillette, and B. B. Brodie. (1973) Acetaminophen-Induced Hepatic Necrosis. I. Role of Drug Metabolism. J. Pharmacol. Exp. Ther. 187:185-194.

Moore, L. (1980) Inhibition of Liver Microsomal Calcium Pump by in vivo administration of Carbon Tetrachloride, Chloroform, and 1,1-

Dichloroethylene. Biochem. Pharmacol. 29:2505-2511.

Moore, L. (1981) Inhibition of Liver Endoplasmic Reticulum Calcium Pump Releases a Pool of Sequestered Calcium. The Pharmacologist. 23:221.

Moore, L., T. Chen, H. R. Knapp, Jr., and E. J. Landon. (1975) Energy-Dependent Calcium Sequestration Activity in Rat Liver Microsomes. J. Biol. Chem. 250:4562-4568.

Moore, L., G. R. Davenport, and E. J. Landon. (1976) Calcium Uptake of a Rat Liver Subcellular Fraction in Response to in vivo Administration of Carbon Tetrachloride. J. Biol. Chem. 251:1197-1201.

Moore, M., H. Thor, G. Moore, S. Nelson, P. Moldeus, and S. Orrenius. The Toxicity of Acetaminophen and N-Acetyl-p-benzoquinone Imine in Isolated Hepatocytes Is Associated with Thiol Depletion and Increased Cytosolic Calcium. J. Biol. Chem. 260:13035-13040.

Morgan, N. G., P. F. Blackmore, and J. H. Exton. (1983) Age-Related Changes in the Control of Hepatic Cyclic AMP Levels by alpha-1 and beta-2 Adrenergic Receptors in Male Rats. J. Biol. Chem. 258:5103-5109.

Murphy, E., K. Coll, T. L. Rich, and J. R. Williamson. (1980) Hormonal Effects on Calcium Homeostasis in Isolated Hepatocytes. J. Biol. Chem. 255:6600-6608.

Orrenius, S. (1986) Recent Studies on the Role of Calcium in Chemical Toxicity. Presentation in Symposium on Mechanisms of Cell Injury, 25th Annual Meeting of the Society of Toxicology, New Orleans, LA.

Newman, J. D., and J. McArmstrong. (1978) On the Activities of Glycogen Phosphorylase and Glycogen Synthase in the Liver of the Rat. Biochim. Biophys. Acta 544:225-233.

Omura, T., and R. Sato. (1964) The Carbon Monoxide Binding Pigment of Liver Microsomes. I. Evidence for its Hemoprotein Nature. J. Biol. Chem. 239:2370-2378.

Pencil, S. D., W. J. Brattin, E. A. Glende, and R. O. Recknagel. (1984) Evidence Against Involvement of Calcium in Carbon Tetrachloride-Dependent Inhibition of Lipid Secretion by Isolated Hepatocytes. Biochem. Pharmacol. 33:2425-2429.

Pencil, S. D., E. A. Glende, Jr., and R. O. Recknagel. (1982) Loss of Calcium Sequestration Capacity in Endoplasmic Reticulum of Isolated Hepatocytes treated with Carbon Tetrachloride. Res. Commun. Chem. Pathol. 36:413-428.

Perrissoud, D., G. Auderset, O. Reymond, and M. F. Maignan. (1981) The Effect of Carbon Tetrachloride on Isolated Rat Hepatocytes: Early Morphological Alterations of the Plasma Membrane. Virch. Arch.

(Cell. Path.) 35:83-91.

Phillips, A. H., and R. G. Langdon. (1962) Hepatic Triphosphopyridine Nucleotide-Cytochrome c Reductase: Isolation, Characterization, and Kinetic Studies. J. Biol. Chem. 237:2652-2660.

Plaa, G. L., and H. P. Witschi. (1976) Chemicals, Drugs, and Lipid Peroxidation. Annu. Rev. Pharmacol. Toxicol. 16:125-141.

Pohl, L. R., R. D. Schulick, R. J. Highet, and J. W. George. (1984) Reductive Oxygenation Mechanism of Metabolism of Carbon Tetrachloride to Phosgene by Cytochrome P-450. Mol. Pharmacol. 25:318-321.

Poli, G., K. Cheeseman, T. F. Slater, and M. U. Dianzani. (1981) The Role of Lipid Peroxidation in Carbon Tetrachloride-Induced Damage to Liver Microsomal Enzymes: Comparative Studies in vitro using Microsomes and Isolated Liver Cells. Chem.-Biol. Interac. 37:13-24.

Poli, G., E. Gravela, E. Albano, and M. U. Dianzani. (1979) Studies on Fatty Liver with Isolated Hepatocytes. II. The Action of Carbon Tetrachloride on Lipid Peroxidation, Protein, and Triglyceride Synthesis and Secretion. Exp. Mol. Pathol. 30:116-127.

Raabo, E., and T. Terkildsen. (1960) On the Enzymatic Determination of Blood Glucose. J. Clin. Lab. Invest. 12:402-407.

Ray, P. and L. Moore. (1986) Carbon Tetrachloride-Induced Release of Calcium From Isolated Rat Hepatocytes. Toxicology. In press.

Recknagel, R. O. (1967) Carbon Tetrachloride Hepatotoxicity. Pharmacol. Rev. 19:145-208.

Recknagel, R. O. (1983) A New Direction in the Study of Carbon Tetrachloride Hepatotoxicity. Life Sci. 33:401-408.

Recknagel, R. O., and E. A. Glende, Jr. (1973) Carbon Tetrachloride Hepatotoxicity: An Example of Lethal Cleavage. CRC Crit. Rev. Toxicol. 2:263-297.

Reinhart, P. H., W. M. Taylor, and F. L. Bygrave. (1984) The Role of Calcium Ions in the Mechanism of Action of alpha-Adrenergic Agonists in Rat Liver. Biochem. J. 223:1-13.

Reynolds, E. S. (1963) Liver Parenchymal Cell Injury. I. Initial Alterations of the Cell Following Poisoning with Carbon Tetrachloride. J. Cell Biol. 19:139-157.

Reynolds, E. S. (1964) Liver Parenchymal Cell Injury. II. Cytochemical Events Concerning the Mitochondrial Dysfunction Following Poisoning with Carbon Tetrachloride. Lab. Invest. 13:1457-1470.

Reynolds, E. S., H. J. Ree, and M. T. Moslen. (1972) Liver Parenchymal Cell Injury. IX. Phenobarbital Potentiation of Endoplasmic Reticulum Denaturation Following Carbon Tetrachloride. Lab. Invest. 26:290-295.

Santone, K. S., and D. Acosta. (1985) Interference of Intracellular Calcium Dynamics in Carbon Tetrachloride Injury of Cultured Rat Hepatocytes. The Toxicologist 5:74.

Sasame, H. A., J. A. Castro, and J. R. Gillette. (1968) Studies on the Destruction of Liver Microsomal Cytochrome P-450 by Carbon Tetrachloride Administration. Biochem. Pharmacol. 17:1759-1768.

Sato, A., and T. Nakajima. (1979) A Structure-Activity Relationship of Some Chlorinated Hydrocarbons. Arch. Environ. Health March/April:69-75.

Schanne, F. A. X., A. B. Kane, E. A. Young, and J. L. Farber. (1979) Calcium Dependence of Toxic Cell Death: A Final Common Pathway. Science 206:700-702.

Schmidt, W. K., and E. L. Way. (1979) Direct Assay for Calcium in Brain Homogenates and Synaptosomal Pellets. J. Neurochem. 32:1095-1098.

Scott, J. A. (1984) Phospholipase Activity and Plasma Membrane Homeostasis. J. Theor. Biol. 111:659-665.

Shatkin, A. J. (1969) Colorimetric Reactions for DNA, RNA, and Protein Determinations. In: Fundamental Techniques in Virology, ed. K. Habel and N. P. Salzman. Academic Press, NY, 231-237.

Shier, W. T. (1982) Cytolytic Mechanisms: Self-Destruction of Mammalian Cells by Activation of Endogenous Hydrolytic Enzymes. J. Toxicol.-Toxin Rev. 1:1-32.

Shier, W. T., and D. J. DuBourdieu. (1982) Role of Phospholipid Hydrolysis in the Mechanism of Toxic Cell Death by Calcium and Ionophore A23187. Biochem. Biophys. Res. Commun. 109:106-111.

Slater, T. F., and B. C. Sawyer. (1971) The Stimulatory Effects of Carbon Tetrachloride and Other Haloalkanes on Peroxidative Reactions in Rat Liver Fractions In Vitro. Biochem. J. 123:805-814.

Smith, M. T., H. Thor, and S. Orrenius. (1981) Toxic Injury to Isolated Hepatocytes is not Dependent on Extracellular Calcium. Science 213:1257-1259.

Smith, M. T., H. Thor, P. Hartzell, and S. Orrenius. (1982) The measurement of Lipid Peroxidation in Isolated Hepatocytes. Biochem. Pharmacol. 31:19-26.

Stacey, N. H., and J. C. Fanning. (1981) Ultrastructural Changes in Isolated Rat Hepatocytes After Incubation with Carbon

Tetrachloride. Toxicology 22:69-77.

Stacey, N. H., and C. D. Klaassen. (1982) Lack of Protection Against Chemically Induced Injury to Isolated Hepatocytes by Omission of Calcium from the Incubation Medium. J. Toxicol. Envir. Health 9:267-276.

Stacey, N. H., H. Ottenwalder, and H. Kappus. (1982) Carbon Tetrachloride-Induced Lipid Peroxidation in Isolated Rat Hepatocytes with Different Oxygen Concentrations. Toxicol. Appl. Pharmacol. 62:421-427.

Stacey, N. H., and B. G. Priestly. (1978a) Dose-Dependent Toxicity of Carbon Tetrachloride in Isolated Rat Hepatocytes and the Effects of Hepatoprotective Treatments. Toxicol. Appl. Pharmacol. 45:29-39.

Stacey, N. H., and B. G. Priestly. (1978b) Lipid Peroxidation in Isolated Rat Hepatocytes: Relationship to Toxicity of Carbon Tetrachloride, ADP/Fe^{++}, and Diethyl Maleate. Toxicol. Appl. Pharmacol. 45:41-48.

Stahlmans, W., H. deWulf, L. Hue, and H.-G. Hers. (1974) The Sequential Inactivation of Glycogen Phosphorylase and Activation of Glycogen Synthetase in Liver after the Administration of Glucose to Mice and Rats. Eur. J. Biochem. 41:127-134.

Suarez, K. A., G. P. Carlson, G. C. Fuller, and N. Fausto. (1972) Differential Acute Effects of Phenobarbital and 3-Methylcholanthrene Pretreatment on Carbon Tetrachloride-Induced Hepatotoxicity in Rats. Toxicol. Appl. Pharmacol. 23:171-177.

Theen, J., D. P. Gilboe, and F. Q. Nuttal. (1982) Liver Glycogen Synthase and Phosphorylase Changes In Vivo with Hypoxia and Anesthestics. Am. J. Physiol. 243:E182-E187.

Toyo-oka, T., K. Hara, N. Nakamura, M. Kitahara, and T. Masaki. Calcium Overload and the Action of Calcium-Sensitive Proteases, Phospholipases, and Prostaglandin E_2 in Myocardial Cell Degradation. Basic Res. Cardiology 80:303-315.

Traiger, G. J., and G. L. Plaa. (1971) Differences in the Potentiation of Carbon Tetrachloride Toxicity in Rats by Ethanol and Isopropanol Pretreatment. Toxicol. Appl. Pharmacol. 20:105-112.

Trump, B. F., I. K. Berezesky, and R. A. Cowley. (1982) The Cellular and Subcellular Characteristics of Acute and Chronic Injury With Emphasis on the Role of Calcium. In: Pathophysiology of Shock, Anoxia, and Ischemia, ed. R. A. Cowley and B. F. Trump. Williams and Wilkins, Baltimore, 6-45.

Tsien, R. Y., T. Pozzan, and T. J. Rink. (1982) Calcium Homeostasis in Intact Lymphocytes: Cytoplasmic Free Calcium Monitored with a New, Intracellularly Trapped Fluorescent Indicator. J. Cell Biol.

94:325-334.

Tsokos-Kuhn, J. O., E. L. Todd, J. B. McMillin-Wood, and J. R. Mitchell. (1985) ATP-Dependent Calcium Uptake by Rat Liver Plasma Membrane Vesicles. Mol. Pharmacol. 28:56-61.

Tyson, C. A., K. Hawk-Prather, D. L. Story, and D. H. Gould. (1983) Correlations of In Vitro and In Vivo Hepatotoxicity for Five Haloalkanes. Toxicol. Appl. Pharmacol. 70:289-302.

Ueng, T.-U., L. Moore, R. G. Elves, and A. P. Alvares. (1983) Isopropanol Enhancement of Cytochrome P450-Dependent Monooxygenase Activities and Its Effects on Carbon Tetrachloride Intoxication. Toxicol. Appl. Pharmacol. 71:204-214.

Uhing, R. J., A. M. Janski, and D. J. Graves. (1979) The Effects of Solvents on Nucleotide Regulation of Glycogen Phosphorylase. J. Biol. Chem. 254:3166-3169.

VanRossum, G. D. V. (1970) Net Movements of Calcium and Magnesium in Slices of Rat Liver. J. Gen. Physiol. 55:18-32.

Waller, R. L., E. A. Glende, Jr., and R. O. Recknagel. (1983) Carbon Tetrachloride and Bromotrichloromethane Toxicity. Biochem. Pharmacol. 32:1613-1617.

Weibel, E. R., W. Staubli, H. R. Gnagi, and F. A. Hess. (1969) Correlated Morphometric and Biochemical Studies on the Liver Cell. J. Cell Biol. 42:68-71.

Widnell, C. C., and J. C. Unkeless. (1968) Partial Purification of a Lipoprotein with 5′-Nucleotidase Activity from Membranes of Rat Liver Cells. Proc. Nat. Acad. Sci. 61:1050-1057.

Williamson, J. R., R. H. Cooper, and J. B. Hoek. (1981) Role of Calcium in the Hormonal Regulation of Liver Metabolism. Biochim. Biophys. Acta 639:243-295.

Winer, B. J. (1971) Statistical Principles in Experimental Design. MacGraw-Hill Company, New York, NY.

Wroblewski, F., and J. S. LaDue. (1956) Serum Glutamic-Pyruvic Transaminase in Cardiac and Hepatic Disease. Proc. Soc. Exp. Biol. Med. 91:569-571.

Wyllie, A. H. (1986) Apoptosis: Cell Death Under Homeostatic Control. Presentation in Symposium on Mechanisms of Cell Injury, 25th Annual Meeting of the Society of Toxicology, New Orleans, LA.